ENHANCED DNA TYPING KITS FOR CHALLENGING FORENSIC DNA SAMPLES

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABI	Applied Biosystems
BSA	Bovine Serum Albumin
CCD	Charge Coupled Device
CE	Capillary Electrophoresis
CSF1PO	CSF-1 receptor (FMS) gene
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
Hi-Di Formamide	Highly Deionised Formamide
FGA	Fibrinogen alpha chain gene
FTA	Flinders Technology Associates
ND	Not Detected
NIST	National Instiute of Standards and Technology
PCR	Polymerase Chain Reaction
PEC	PCR Enhancing Cocktail
POP-4	Performance Optimized. Polymer 4
RFLP	Random Fragment Length Polymorphism
RFU	Relative Fluorescent Units
SRM	Standard Reference Material
SRY	Sex-Determining Region Y
STR	Short Tandem Repeats
SWGDAM	Scientific Working Groups on DNA Analysis Methods
T	Thymine

Taq	Thermus Aquaticus
TH01	Intron 1 of the Thyrosine Hydroxylase Gene
TPOX	Human Thyroid Peroxidase Gene
U	Uracil
USER	Uracil-Specific Excising Reagent
VNTR	Variable Number of Tandem Repeats

LIST OF TABLES AND FIGURES

ABSTRACT

Degradation in forensic DNA samples, reliable gender determination and inhibition of the polymerase chain reaction (PCR) process are the main challenges to DNA typing. Using a combination of a Taq mutant polymerase (OmniTaq), EzWay™ PCR Direct Buffer, panel of gender determining markers and reduced-size Short Tandem Repeat (STR) primer sets, developmental validation using Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines were tested on two miniplexes. Miniplex 1 comprises of the larger STR loci in the AmpF*l*STR® Identifiler® PCR Amplification kit (D2S1338, D21S11, CSF1PO, D7S820, D13S317, TPOX, D18S51, D16S539 and FGA) and three gender markers: sex-determining region Y (SRY), Amelogenin and DYS392. Miniplex 2 comprises of the remaining STR loci (TH01, D19S433, D13S317, D3S1358, D2S1776, D5S818, vWA, D8S1179) and two additional STR markers D2S1776 and DYS390. Our results demonstrate that the two miniplexes are highly robust in overcoming PCR inhibitors, provide accurate gender determination and useful in the analysis of degraded DNA. A novel method of a single amplification/detection of Miniplex 1 and Miniplex 2 in a single PCR is also presented.

INTRODUCTION

1. Forensic DNA Typing

Forensic science has widely embraced Polymerase Chain Reaction (PCR) based testing as the molecular diagnostic tool of choice today. Technologies used for performing forensic DNA analysis have advanced in the last 20 over years. From ABO blood group determination, to single-locus and multi-locus probe Restricted Fragment Length Polymorphsim (RFLP) methods, the more recent PCR technique has improved in terms of processing time and sensitivity and has moved from requiring huge amount of biological material with intact DNA, to tiny amounts of sample to yield a complete DNA profile (Brettell *et al.* 2009, Butler, 2006).

DNA fingerprinting or DNA typing (profiling) was first discovered in 1985 by a Bristish geneticist named Alec Jeffreys who described the repeated DNA sequence variations among human individuals which could be used for human identification. The basis of these sequence variations among human individuals permits techniques to be developed which allows examination of their length variations. These DNA repeats regions or minisatellites, became known as variable number of tandem repeats, also known as VNTR can range from 9-80 bp. Minisatellites resides in non-coding regions of the human genome which could range from 1kb 20 kb in length. The technique to examine the length of the repeat sequence varations employed the use of restriction enzymes, thereby earning the name of RFLP (Jeffreys *et al.* 1985).

Today, instead of VNTR, short tandem repeats (STRs), also characterised as microsatellites, are widely used in human identity testing. These genetic markers contain repeated sequences of 2-6 base pairs in length arranged in tandem. STRs are highly polymorphic, but with the ease of genotyping by using multiplex PCR (Moretti *et al.*

2001, Lygo *et al.* 1994 Edwards *et al.* 1991, Hammond *et al.* 1994, Budowle *et al.* 1999) they can generate small amplicons that can be rapidly separated in automated detection of fluorescent-labeled PCR products after capillary electrophoresis (CE). The small amplicon size of STR alleles in contrast to minisatellites also means that STRs are suitable in the analysis of degraded DNA commonly encountered in forensic samples (Hummel *et al*. 1999, Alonso *et al.* 2001, Takahashi *et al.* 1997, Whitaker *et al.* 1995, Clayton *et al.* 1995).

As a result, this has led to the prevalence of use of STRs in forensic DNA typing. Consequently, National DNA databases to assist in criminal and missing persons investigation was introduced in several countries, first in the United Kingdom and subsequently in United States. Each national DNA database adopts a fixed set of STR markers. In the United States, 13 of these STR markers are selected by the Scientific Working Group to DNA Analysis Methods (SWGDAM) (Budowle *et al.* 1998). These markers are D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, vWA, TPOX, D18S51, D5S818 and FGA and are used across all federal, state and community forensic testing laboratories throughout the United States who contributes to their National Databases, also known as the Combined DNA Index System (CODIS). These markers that are selected for the US National Database are also known as the CODIS core loci. The adoption of the 13 core loci for CODIS in the United States has led to commercial companies such as Applied Biosystems (ABI) and Promega to develop STR multiplexes that cover these STR makers (Krenke *et al.* 2002, Holt *et al.* 2002 and Wallin *et al.* 2002) and has also dictated forensic laboratories in the world to adopt the same STRs for use.

2. Challenges in forensic DNA typing

Forensic DNA analysis has to deal with less than ideal DNA samples. The collected biological material, whether in the form of a biological stain from a crime scene, or a highly decomposed body from a homicide, are often left exposed in harsh environment over prolonged periods of time. In addition, biological material recovered from victims of disasters such as from aeroplane crashes, the 2004 Asian Tsunami, the 911 World Trade Centre, earthquakes, landslides or incidents of similar nature, are exposed to sudden extremities of high heat, moisture and other environmental assaults (Holland *et al.* 2003, Sajantila *et al.* 1991, Copeland, 1985, Bohnert and Rothschild, 2003). Tiny amounts of DNA may also be found in highly putrefied bodies (Hoff-Olsen *et al,* 1999, Deng *et al.* 2005), formalin preserved tissues (Budimlija *et al.,* 2005, Turrina *et al.* 2008) or hair shafts (Pfeiffer *et al.* 1999). Body fluids such as blood and semen can also be found on soil, sand, wood, leaf litter, dyed textile and leather that contain substances which may co-extract during DNA isolation and prevent PCR amplification. Therefore, far from being preserved in an ideal environment such as a freezer, away from physical, chemical and biological elements that can break down the DNA molecules, forensic samples that are collected can present challenges on multiple fronts. The major of which are DNA degradation, PCR inhibitors and anomalous amelogenin genotypes.

3. Degraded DNA

Degradation in forensic DNA samples is a result of environmental exposures, which randomly breaks the DNA into fragments. The culprits of DNA degradation include water, nucleases or other physical and oxidation processes. When template DNA

degradation occurs, the chances of finding a target sequence for both the forward and reverse primers to bind simultaneously for full DNA extension during PCR is greatly reduced. Without a target DNA that flanks the STR repeat region to serve as template, PCR will not be successful because primer extension cannot continue at the break in the template DNA. The more the DNA sample is degraded, the more break points occur in the target DNA resulting in diminishing DNA targets available having the required length for PCR amplification.

To address this problem, reduced-size STR primer sets have been designed (Butler *et al.* 2003). Multiplexes having these primer sets are known as Miniplexes and the primers are designed to bind as close to the repeat region as possible. As commercial multiplex kits such as ABI AmpF*l*STR® Identifiler® and Promega Powerplex® 16 multiplex kits are designed to accommodate multiple markers, a number of primers are designed to generate large amplicons, and the primers are required to move further away from the core repeat sequence. Therefore, when degraded DNA are encountered, DNA typing using commercial multiplex kits result in incomplete or partial DNA profiles, which compromise the strength of the DNA evidence for a affirmative identification. The redesigned primer sets reduce the amplicons size by requiring smaller DNA targets, thereby ensuring greater success in DNA typing.

However, the mini-STR strategy has its drawbacks. One of which is the danger of placing primers close to the repeat region if insetion/deletions occur in the flanking regions of the STR markers but outside of the miniSTR primer binding sites. This will result in different allele calls with different primer set or null alleles and this phenomenon has been observed in D13S317 (Butler *et al.* 2003, Boutrand *et al.* 2001) and D8S1179

(Budowle *et al.* 2001, Han *et al.* 2001). Another is that a few loci can be simultaneously amplified as most of the amplicon spans overlapped between 71 to 250 bp in length. Since the amplicon products of mini-STRs will overlap in size more so than those in conventional STR kits, the four fluorescent dye label system has since been increased to five dyes. This has resulted in more mini-STRs being accommodated into one multiplex system. However when a degraded, limited-quantity DNA sample is present, the limitation prevents the amplification of adequate STR markers like conventional STR kits, which can amplify multiple STR markers in one amplification. Multiple amplifications are required to accomplish the same level of discrimination power of commercial STR typing kits which routinely co-amplifies 16 markers in one PCR.

4. **PCR Inhibition**

Due to the nature of the forensic samples, the extracted DNA is highly vulnerable to the presence of PCR inhibitors from the environment. PCR inhibitors generally exert their effects either by direct interaction with DNA or inactivation of Taq DNA polymerase thus preventing successful amplification (Wilson, 1997). Direct binding of the inhibitors to the DNA can co-purify the inhibitors with the DNA during extraction and prevent PCR amplification. Taq DNA polymerase requires Mg^{2+} as a critical enzyme cofactor and any substance that reduce availability of Mg^{2+} or interfere the binding of Mg^{2+} to the DNA polymerase will inhibit PCR. Important and common source of inhibitors include hematin from blood (Akane *et al.* 1994), humic acid in soil (Tsai and Olson, 1992, Watson and Blackwell, 2000), denim, textile dyes (Shutler *et al.* 1999), tannic acid in leather, decomposing vegetitive material, melanin in hair (Echkart *et al.* 2000), polysaccharides

and bile salts in feces (Monteiro *et al.* 1997) and urea in urine (Mahony *et al.* 1998). The result of amplification in the presence of inhibitor is a loss of the alleles from the larger size STR markers or even amplification failures of all STR markers. Samples having PCR inhibitors generate partial DNA profiles that look identical to a degraded DNA sample. This is due to smaller PCR products being more efficiently amplified than the larger one under inhibitory PCR conditions.

There are strategies proposed to overcome PCR inhibitors and reviews of those approaches have been published (Wilson 1997, Rådström *et al.* 2004). PCR inhibitors can either be removed or their effects diminished by the following solutions. The target DNA can be diluted which also reduce the concentration of the PCR inhibitors, allowing amplification. Alternatively, adoption of DNA extraction protocols that efficiently extract inhibitor-free DNA such as the use of sodium hydroxide (Bourke *et al.* 1999), the addition of aluminium ammonium sulfate (Braid *et al.* 2003), or the use of purification steps like Centricon-100 and Microcon-100 filters (Comey *et al.* 1994) and low-melt agarose gel plugs (Moreira 1998) have been used to separate the inhibiting compounds from the extracted DNA. There are also commercial DNA extraction kits such as Applied Biosystems Prepfiler™ or Promega DNA IQ™ that have staked their effectiveness in removing PCR inhibitors but these will require extensive validation testing by the laboratory. Additives to the PCR reaction, such as bovine serum albumin (BSA) (Comey *et al.* 1994) are found to be able to partially overcome the effects of PCR inhibitors by either stabilizing the DNA polymerase or by binding the inhibitors. Betaine (Al-Soud and Rådström 1998) and the single-stranded DNA binding protein of the T4 32 gene (Kreader 1996) have also been shown to prevent or minimise the inhibition of PCR but

these are inhibitor-specific in nature. Non-Taq DNA polymerase such as rTth, Tfl, HotTub and Pwo, can tolerate higher concentrations of blood and feces, which typically inhibit PCR when performed with Taq DNA polymerase (Al-Soud and Rådström 1998). More recently, alternative DNA polymerases-buffer systems with higher tolerance to PCR inhibitors compared to Taq polymerase have been demonstrated (Park *et al.* 2005, Barbaro *et al.* 2008, Hedman *et al.* 2009).

5. **Amelogenin Deletions**

Gender identification or gender typing is commonly performed together with STRs in commercial kits using PCR products generated only from the amelogenin gene that occurs on both the X- and Y-chromosome. A commonly used PCR primer set published by Sullivan et al. (1993) targets a 6 bp deletion that occurs on the X-chromosome, which results in the X- and Y-chromosome PCR amplicon size to be differentiated from one another when electrophoretic separation is performed to separate STR alleles. Since females are XX, only a single peak is observed when testing female DNA whereas males, which possess both X and Y chromosomes, exhibit two peaks with a standard amelogenin test. However, there have been multiple reports in the literature for anomalous amelogenin results due to primer binding site mutations (Roffery *et al.* 2000, Shewale *et al.* 2004. Shadrach *et al.* 2004, Alves *et al.* 2006) or deletions of sections of the Y-chromosome (Santos *et al.* 1998, Steinlechner *et al.* 2002, Thangaraj *et al.* 2002, Michael and Brauner 2004, Lattanzi *et al.* 2005, Mitchell *et al.* 2006, Santacroce *et al.* 2006, Cadenas *et al.* 2007, Chen *et al.* 2007, Chang *et al.* 2007, Yong *et al.* 2007, Jobling *et al.* 2007, Kumagai *et al.* 2008). The results of which could mislead either crime investigators

into believing a female perpetuator is involved, or with highly decomposed or fragmented or human remains, the gender be falsely identified as a female. The frequency of these cases are reported to be low among Caucasians but are found to reach significant levels in several other populations (Chang *et al.* 2003, Lim *et al.* 2004, Kashyap *et al.* 2006, Chang *et al.* 2007). With the investigative impact of the gender of a sample so important, other additional gender markers e.g. SRY (Santos *et al.* 1998), DXYS156 (Cali *et al.* 2002), Y-STRs (Chang *et al.* 2007) or alternative amelogenin primer sets (Haas-Rochholz and Weiler 1997) have been proposed to complement the amelogenin marker.

6. **Aims**

Given the myriad of challenges that forensic DNA typing analysis possess, our laboratory is interested in developing multiplex kits that can simultaneously address the problems of DNA degradation, PCR inhibition and anomalous amelogenin typing which at present is unavailable in any commercial DNA typing assays. Developmental validation studies of the multiplex assay were undertaken in accordance with the Scientific Working Group to DNA Analysis Methods (SWGDAM guidelines (http://www.fbi.gov/hq/lab/fsc/backissu/july2004/standards/2004_03_standards02.htm). These guidelines require a series of tests for the laboratory to assess the limitations of an analysis method, and to examine the different parameters that could affect the ability of the method to produce reliable results under a variety of conditions (Lygo *et al.* 1994). A series of tests were performed that includes concordance with standard multiplex kits, sensitivity, reproducibility and PCR amplification conditions. In addition, studies of DNA mixtures, non-human DNA testing, degraded DNA samples and studies involving

both simulated forensic samples and casework were covered. This study will cover the development strategies employed, and the various tests that are performed as spelt out by SWGDAM, which will demonstrate the limits and strengths of this approach to overcome the several challenges faced by current forensic DNA typing. Additionally, a novel forensic DNA typing strategy is also introduced which enabled more DNA typing results when limited highly degraded DNA is encountered.

The objective of this project is to develop a method which will benefit justice by rendering useful DNA profiles from a significantly high percentage of forensic samples in human identity testing, which are challenged by degradation, PCR inhibition and gender mis-typing during forensic DNA typing.

MATERIALS AND METHODS

1. Design and development of Miniplex 1 and Miniplex 2

1.1 Locus selection and characterization

Miniplex 1 (Fig. 1) comprised the larger STR loci in the AmpF*l*STR® Identifiler® PCR Amplification kit (D2S1338, D21S11, CSF1PO, D7S820, D13S317, TPOX, D18S51, D16S539 and FGA) and three gender markers: sex-determining region Y (SRY), Amelogenin and DYS392. Miniplex 2 (Fig 2) comprised the remaining STR loci (TH01, D19S433, D13S317, D3S1358, D2S1776, D5S818, vWA, D8S1179) and two additional STR markers D2S1776 and DYS390. Both Miniplex 1 and Miniplex 2 have D13S1358 and serve as a genotype concordance between both Miniplexes to monitor potential sample mix-up between amplifications. All the STR markers have been characterised extensively for phyisical linkage, Mandelian inheritance, approximation of Hardy-Weinberg equilibrium and independent assortment (Budowle *et al.* 1998; Cotton *et al.* 2000; Budowle *et al.* 2001; Hill *et al.* 2008).

The PCR primer sequences for the selected STR loci and gender-typing markers were taken from published literature (Table 1 and Table 2) and the primers were selected due to their design to be as close as the STR target region as possible and termed as miniSTR primers (Butler *et al.* 2003). The exceptions were vWA, D13S317 and SRY. The final primers combination in Miniplex 1 and Miniplex 2 were tested for potential binding issues with each other using AutoDimer (Vallone and Butler, 2003). All of the forward primers were labeled with either 6FAM™ (blue), VIC® (green), NED™ (yellow), or PET™ (red) fluorescent dyes, and with the LIZ® dye used to label the GeneScan™-500

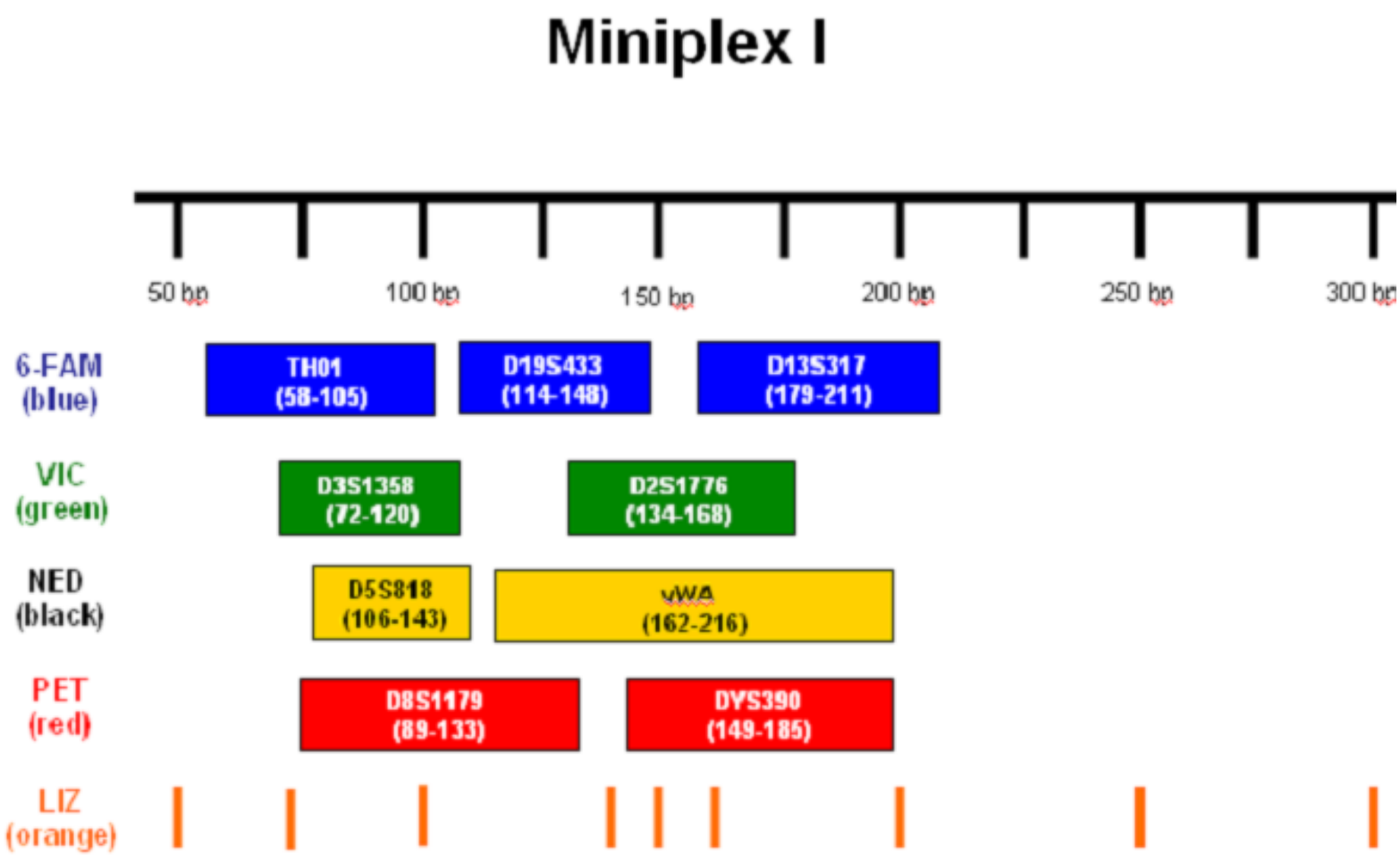

Figure 1. Schematic layout of Miniplex 1 kit for a single amplification of 10 autosomal STR markers, 1 Y-STR marker and 2 gender-determining SRY and amelogenin markers. General size ranges and dye-labelling strategies are indicated.

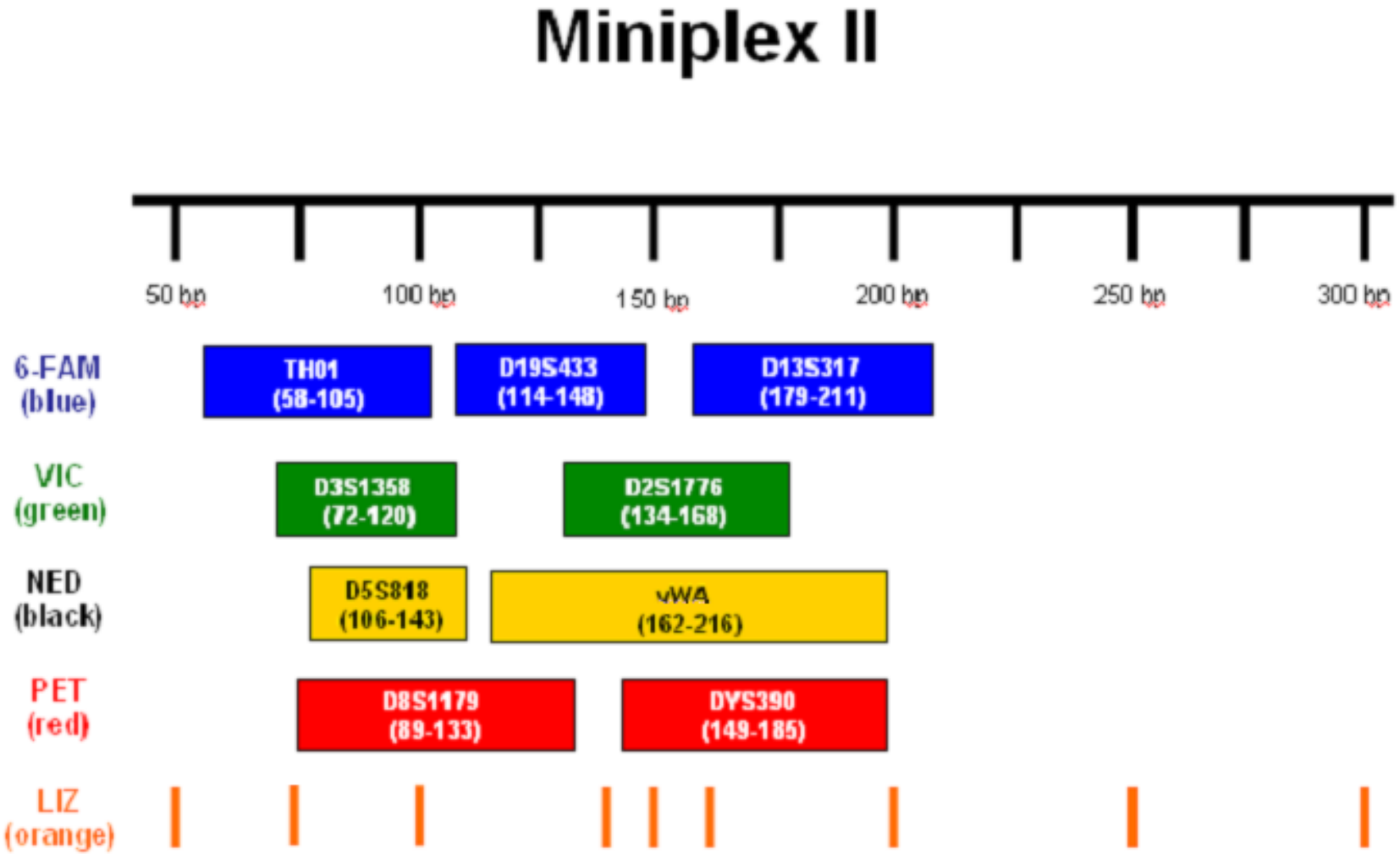

Figure 2. Schematic layout of Miniplex 2 kit for a single amplification of 8 autosomal STR markers and 1 Y-STR marker. General size ranges and dye-labelling strategies are indicated.

Table 1. Primer sequences and assay concentration used in Miniplex 1.

Miniplex I

Locus Name		Forward Dye Label	PrimerSeq(5'to3')	Primer Concentration, µM	Reference
SRY	F	6-FAM	GTATCGACCTCGTCGGAAG	0.5	This study
	R		GAGTACCGAAGCGGGATCT	0.5	This study
Amelogenin	F	6-FAM	CCTTTGAAGTGGTACCAGAGCA	0.5	Haas-Rochholz and Weiler, 1997
	R		GCATGCCTAATATTTTCAGGGAA	0.5	Haas-Rochholz and Weiler, 1997
D2S1338	F	6-FAM	TGGAAACAGAAATGGCTTGG	0.5	Butler *et al.*, 2003
	R		CATTGCAGGAGGGAAGGAAG	0.5	Butler *et al.*, 2003
D21S11	F	6-FAM	ATTCCCCAAGTGAATTGC	7.25	Butler *et al.*, 2003
	R		GGTAGATAGACTGGATAGATAGACGA	7.25	Butler *et al.*, 2003
DYS392	F	6-FAM	ACCTACCAATCCCATTCCTT	1	This study
	R		**ATT**CTGTAAATGGTTGTATAGTATTTTATG	1	This study
CSF1PO	F	VIC	ACAGTAACTGCCTTCATAGATAG	1	Butler *et al.*, 2003
	R		GTGTCAGACCCTGTTCTAAGTA	1	Butler *et al.*, 2003
D7S820	F	VIC	GAACACTTGTCATAGTTTAGAACGAAC	0.5	Butler *et al.*, 2003
	R		**ATTT**CATTGACAGAATTGCACCA	0.5	Butler *et al.*, 2003
D13S317	F	VIC	GGCAGCCCAAAAAGACAGA	0.25	Krenke *et al.*, 2002
	R		**ATTATTATT**ACAGAAGTCTGGGATGTGGAGGA	0.25	Krenke *et al.*, 2002
TPOX	F	NED	CTTAGGGAACCCTCACTGAATG	1	Butler *et al.*, 2003
	R		**ATTT**TGTCCTTGTCAGCGTTTATTTGC	1	Butler *et al.*, 2003
D18S51	F	NED	TGAGTGACAAATTGAGACCTT	0.5	Butler *et al.*, 2003
	R		**ATTATT**GTCTTACAATAACAGTTGCTACTATT	0.5	Butler *et al.*, 2003
D16S539	F	PET	ATACAGACAGACAGACAGGTG	3	Butler *et al.*, 2003
	R		GCATGTATCTATCATCCATCTCT	3	Butler *et al.*, 2003
FGA	F	PET	AAATAAAATTAGGCATATTTACAAGC	4	Butler *et al.*, 2003
	R		GCTGAGTGATTTGTCTGTAATTG	4	Butler *et al.*, 2003

Table 2. Primer sequences and assay concentration used in Miniplex 2.

Miniplex 2

Locus Name		Forward Dye Label	PrimerSeq(5'to3')	Primer Concentration, µM	Reference
TH01	F	6'FAM	CCTGTTCCTCCCTTATTTCCC	0.5	Butler *et al.*, 2003
	R		**ATTT**ACAGGGAACACAGACTCCATG	0.5	Butler *et al.*, 2003
D19S433	F	6'FAM	CCTGGGCAACAGAATAAGAT	2	This study
	R		**ATTATTATT**CCCGAATAAAAATCTTCTCTCTTTC	2	This study
D13S317	F	6'FAM	GGCAGCCCAAAAAGACAGA	2.5	Krenke *et al.*, 2002
	R		**ATT**ACAGAAGTCTGGGATGTGGAGGA	2.5	Krenke *et al.*, 2002
D3S1358	F	VIC	CAGAGCAAGACCCTGTCTCAT	1	Butler *et al.*, 2003
	R		**ATT**TCAACAGAGGCTTGCATGTAT	1	Butler *et al.*, 2003
D2S1776	F	VIC	TGAACACAGATGTTAAGTGTGTATATG	4	Hill *et al.*, 2008
	R		**ATTATTATT**TCTGAGGTGGACAGTTATGAAA	4	Hill *et al.*, 2008
D5S818	F	NED	GGGTGATTTTCCTCTTTGGT	2	Butler *et al.*, 2003
	R		**ATT**AACATTTGTATCTTTATCTGTATCCTTATTTAT	2	Butler *et al.*, 2003
vWA	F	NED	GGACAGATGATAAATACATAGGATGGATGG	1	Krenke *et al.*, 2002
	R		**ATT**AGAGGATCCAAGTTGACTTGGCTG	1	This study
D8S1179	F	PET	TTTGTATTTCATGTGTACATTCGTATC	1.5	Butler *et al.*, 2003
	R		**ATT**ACCTATCCTGTAGATTATTTTCACTGTG	1.5	Butler *et al.*, 2003
DYS390	F	PET	CTGCATTTTGGTACCCCATA	4	Park *et al.*, 2007
	R		**ATT**GCAATGTGTATACTCAGAAACAAGG	4	Park *et al.*, 2007

Size Standard (Applied Biosystems, Foster City, CA). The reverse primers (AIT Biotech, Singapore) were unlabeled, with some having an additional ATT or ATTT sequence or a concatamer of 2 to 3 ATT blocks added to the 5' end to promote full adenylation (Krenke *et al.* 2002) (Table 1 and Table 2). The use of the concatamer sequence was to create sufficient non-overlapping spacing in between loci of the miniplex. The final target concentration of the forward and reverse was empirically adjusted to generate balanced PCR products as measured with the Applied Biosytems (ABI) PRISM® 3100 and is shown in Table 1 (Miniplex 1) and Table 2 (Miniplex 2).

1.2 Sample source and extraction protocols

A set of 251 blood samples stained on FTA card with self-identified ethnicities, including 83 Chinese, 78 Malays, 90 Indians obtained from annoymous donors were used in population concordance studies. Whole blood samples from 11 annoymous donors were used in this study. They were extracted for DNA using phenol/choroform (Maniatis *et al.* 1982), which is also known as the organic extraction method. The DNA extract was further purified and concentrated and purified using Mircrocon® YM-100 filters (Millipore Coporation, Bedford, MA). The DNA was quantified using the Quantifiler® Human DNA Quantitiation Kit (Applied Biosystems) and diluted to concentration of 500pg/µl. For the validation studies, genomic 9948 DNA (Promega Corporation, Madison, WI) were used when unspecified. Genomic DNA from male donor "R120" and "R60" are also used in the validation studies and when used, it would be specified.

1.3 PCR Reaction Components and Thermal Cycling Parameters

To determine the suitable range of conditions, several amplification parameters were used. They included *Taq* enzyme concentration, annealing temperature, reaction volume and cycle number. 1 to 5 U of OmniTaq (DNA Polymerase Technology, Inc, St Louis, MO) in 15µl of PCR volume were tested with 500pg of DNA template for Miniplex 1 and Miniplex 2, respectively. Annealing temperatures of 55, 57, 58, 59 and 60^{o}C and extension temperatures of 65, 68, 70, 72, 74 and 76 ^{o}C were tested on Miniplex 1 to establish the optimum temperature for PCR. Reaction volumes of 5, 10, 15, 20, 25, 50 µl were tested with 500 pg of DNA. The 500pg of DNA template were amplified at 28, 30 and 32 PCR cycles. The testing was done in triplicates. In order to promote adenlylation and increase the yield of PCR products of Miniplex 2, a series of NaOH concentrations from 0.013, 0.020, 0.026, 0.033 to 0.040 M were added during PCR. The final PCR reaction components that were used are as follows: 1X Primer Mix, 200µM of each dNTP, 1X EzWay^{TM} Direct PCR Buffer (Komabiotech, Seoul, Korea) and 1U/15µl OmniTaq (DNA Polymerase Technology, St Louis, MO). For Miniplex 2, 0.026M of NaOH was included in the PCR reaction. Thermal cycling parameters used were 96^{o}C/2min, followed by 94^{o}C/1min, 59^{o}C/2min, 74^{o}C/1min, for 10 cycles, 90^{o}C/1min, 59^{o}C/2min, 74^{o}C/1min for 20 cycles and 60^{o}C/90min.

1.4. Removal of residual dyes after PCR

In order to remove residual dye molecules that resulted in “dye blobs” during capillary electrophoresis electropherograms and to increase capillary electrophoresis signal levels, MinElute spin columns (Qiagen, Inc. Valencia, CA) or MinElute 96 UF PCR Purification kit (Qiagen, Inc. Valencia, CA) were used to “clean-up” the PCR products before genotyping on

the ABI PRISM® 3100. A total of 15 µl of the PCR products were processed according to the manufacturer's protocol and the PCR products were eluted in 15µl of EB buffer. Whenever the amplification was performed on 0.2ml PCR tubes, MinElute spin columns were used, and when PCR was performed on 96-well PCR plates, MinElute 96 UF PCR plates were used instead.

1.5 Analysis on the ABI PRISM® 3100 Genetic Analyzer

Amplification products were diluted in Hi-Di formamide (Applied Biosystems) by adding 1 µl PCR product and 0.3 µl GS500-LIZ internal size standard (Applied Biosystems) to 8.7 µl of Hi-Di. The samples were analysed on the 16-capillary ABI Prism 3l00 Genetic Analyzer after denaturation of samples at 95^{o}C for 3 min and snap-cooled at -20^{o}C for 3min. Prior to testing, a 5-dye matrix was established under the ''G5 filter'' with the five dyes of 6FAM, VIC™, NED™, PET™, and LIZ™. Samples were injected electrokinetically for 10 sec at 3 kV. The STR alleles were then separated at 15 kV at a run temperature of 60^{o}C using the POP-4™ (Applied Biosystems) and 1X Genetic Analyser Buffer with EDTA and on a 36 cm array (Applied Biosystems). Data from the ABI PRISM® 3100 were analysed using GeneScan® Software 3.7 (Applied Biosystems) with peak amplitude threshold set at 50 relative fluorescence units (RFU) for all colors. Genotypes were generated using Genotyper® v3.7 (Applied Biosystems).

1.6 Generation of Allelic Ladder and Genotyper Macros

Allelic ladders for the autosomal STRs in Identifiler™ (Applied Biosystems) and Y-STRs were created using a 1:1000 dilution of allelic ladders from the Identifiler™ or Yfiler™ (Applied Biosystems), respectively. For each STR locus, 2 µl of the diluted ladders were

amplified using 1X GoldST*R buffer (Promega, Corporation, Madison, WI), 2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosytems) and 1µM of primer for each STR locus in reaction volumes of 15 µl at 20 cycles using the thermal cycling parametres as described by Butler *et al.* 2003. D2S1776 allele ladder was generated using a combination of individual samples that represent each allele commonly observed in the population data sets. The samples were amplified by pooling 500 pg of DNA from each sample in a single PCR for 30 cycles using the same PCR conditions. Similarly for SRY and amelogenin, alleles were obtained by amplifying 500pg of 9948 (Promega) for 30 cycles. 1µl of amplified PCR products for each marker were analysed on the ABI PRISM® 3100 Genetic Analyser (Applied Biosytems) to determine the signal level. Varying amounts of PCR products of each marker were mixed to generate a balanced signal level (~200 to 300 RFUs) in the combined allelic ladder for Miniplex 1 and Miniplex 2 using the MinElute spin column (Qiagen) and eluted using a 50µl volume. Genotyper macros were constructed for Miniplex 1 and Miniplex 2 to work with the new allelic ladders. 1µl of the allelelic ladder was used as reference for each genotyping analysis.

2 Validation studies for Miniplex 1 and Miniplex 2

2.1 Sensitivity Studies

In order to assess the performance and interpretation guidlelines of Miniplex 1 and Miniplex 2, varying amount input DNA template and its impact in generating a DNA profile was assessed in PCR amplifications. Triplicate amplifications for Miniplex 1 and five repeated amplifications for Miniplex 2 were performed on a dilution series of a genomic sample (1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.15625 ng). The heterozygous peak balance ratio at all six

DNA amounts was also calculated. Only samples that were heterozygous for a particular locus were included in the calculations. Threshold for detection was set at 25 RFUs for this study to obtain more data for the calculation. The peak balance ratio was calculated by dividing the peak height of the smaller peak by the peak height of the larger peak. For samples with complete dropout of one allele, a zero peak balance ratio was assigned.

2.2 Stutter calculations

To ensure reliable genotyping, intepretation guidelines to distinguish true alleles from stuter artefacts generated during PCR are reuqired. Peak heights of stutters and its allele peaks were exported from Genotyper® v3.7 (Applied Biosystems) software into Microsoft® Excel. The stutter percent was calculated by taking the peak heights of the stutters and dividing over the peak height of its allele peaks and expressed as a percentage. The average and highest stutter percentage was noted and used as stutter percentage threshold in determining a true allele peak from an artefact stutter.

2.3 Stability Studies

To determine PCR efficiency of Miniplex 1 and Miniplex 2 in the presence of varying concentrations of inhibitors, porcine hematin (Sigma Aldrich, St. Louis, MO), a heme-containing known inhibitor, was added to 500 pg of input DNA and amplified with 50 µM increments in concentrations, from 0 µM to 500 µM and performed in triplicates for both Miniplex 1 and Miniplex 2. Similary, tannic acid (Sigma Aldrich, St. Louis, MO) and humic acid (Sigma Aldrich, St. Louis, MO), which are known inhibitors from leather and soil, respecitively were aded in 50 µM increments in concentrations, from 0 µM to 300 µM to 500 pg of input DNA prior to amplification.

2.4 Species Specificity

To determine that Miniplex 1 and Miniplex 2 demonstrate spcificity for human DNA, a variety of animal and microbe DNA were examined. Primate DNA samples with known quantity were obtained from Dr. Rolf Meier (Department of Biological Sciences, NUS, Singapore) and various non-primate and primate blood samples were obtained from the Forensic Chemistry and Phyiscs Laboratory (FCPL), HSA, Singapore. The animal blood samples were stained on FTA cards. Microbial DNA that had been quantified was obtained from Dr. Sanjay Swarup and Dr. Lim Tit Meng (Department of Biological Sciences, NUS, Singapore) and extracts of microbes from decomposing material were directly amplified. 10ng of liquid DNA template or 1.2mm FTA punch were used for PCR amplification.

2.5 Mixture Studies

Two male genomic samples were mixed with the total DNA input fixed at 500pg for amplification and represented in proportions as follows: 19:1, 9:1, 3:1, 1:1, 1:3, 1:9, and 1:19. Amplifications for both Miniplex 1 and Miniplex 2 were performed in triplicates. This was to establish the sensitivity level of Miniplex 1 and Miniplex 2 by which a minor DNA contributor could be detected.

2.6 Degraded DNA Studies

To evaluate the efficency of amplification in the presence of degraded DNA, dexoyribonulease or Dnase I (New England Biolabs, Ipswich, MA) was used to digest DNA for 0, 2, 5, 10, 15 and 20min. 2ng of DNA from each timepoint were added for amplification using Miniplex 1 and Identifiler™ (Applied Biosystems). The performance of the two

multiplex kits was compared to determine the effciency of Miniplex 1 with Identifiler™. Samples were amplified using Identifiler™ in using the protocol specified by manufacturer for 28 cycles.

2.7 Concordance Studies

Samples used for genotyping concordance verification were those included in the National Institue of Standards and Technology Standard Reference Material® 2391b (NIST, Gaithersburg, MD). Sources of DNA also included female 9947A, 9948 male DNA (Promega Corporation, Madison, WI) and 007 male DNA (Applied Biosystems), which were used for initial testing of protocols and positive controls for PCR.

For population concordance, a total of 741 blood samples that were stained on FTA card with self-identified ethnicities were used. These were made up of 249 Chinese, 244 Malays, and 248 Indians from annoymous donors for comparisons with the genotypes generated using the commercial DNA typing STR kit Identifiler™ (Applied Biosystems) to the genotypes developed from Miniplex 1 and Miniplex 2 primer sets. The genotypes using the Miniplex 1 and 2 were developed using a different PCR conditions and components from an earlier study. PCR was performed using 1X GoldST*R buffer (Promega, Corporation, Madison, WI), 2.5 µg BSA (New England Biolabs, Ipswich, MA), 2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosytems) and 1.5 µl of either Miniplex 1 or Miniplex 2 primer set in reaction volumes of 15 µl at 30 cycles using the thermal cycling parametres as described by Butler *et al.* 2003.

In order to verify that concordant genotypes were developed with the new DNA polymerase and PCR buffer systems but with identical Miniplex 1 and Miniplex 2 primer

sets, a subset of 83 Chinese, 78 Malays and 90 Indians samples from the 720 samples were genotyped.

2.8 Population Studies

The STR markers in Miniplex 1 and Miniplex 2 overlapped with the markers used in Identifiler™ (Applied Biosystems) and Yfiler™ (Applied Biosytems), and the allele frequecies of the overlapped markers have been established (Ang *et al.* 2005, Budowle *et al.* 2009, Lim *et al.* 2005, Syn *et al.* 2005) and was not compiled for this study. Only the allele frequency of the non-overlapping D2S1776 STR marker was analysed using PowerStats v12 spreadsheet (http://www.promega.com/geneticidtools/powerststs/).

2.9 Casework Studies

Quantified DNA extracts using organic extraction method from the laboratory internal validation studies on the Maxwell® 16 system (Lim *et al.* 2009) in comparison to phenol/chloroform DNA extraction technique was obtained. Liquid blood from 5 anoynomous donors stained on a variety of substrate to mock as casework samples. The DNA was extracted using organic extraction and purified using Mircrocon® YM-100 filters (Millipore Coporation, Bedford, MA). The following substrates were used, white cotton, blue denim, leather belt and soil. A total of 9 cigarette butts from anoynomous donors were obtained and DNA was isolated using organic extraction. Serial dilutions of the 5 blood samples ranging from neat to 1:500 dilutions were also prepared and the DNA was then extracted and quantified.

Quantitated DNA extracts using organic extraction from four different completed external proficiency test samples comprising of 16 samples were obtained. DNA extracts from 9 blood sample references left from adjudicated casework samples and DNA extracts from 3 completed casework samples were used. To evaluate the performance of Miniplex 1 and Miniplex 2, the DNA profiles that had been obtained using Identifiler™ (Applied Biosystems) were then compared.

3 Initial development of Miniplex C

3.1 Design strategy and protocol for Miniplex C

In order to co-amplify Miniplex 1 and Miniplex 2 primer sets together in one single PCR, instead of 2 separate amplifications, a novel strategy was explored. An internal Thymine (T) nucleotide was selected and subsituted with Uracil (U) nucleotide in the dye-labelled forward primers in Miniplex 1. Similarly, an internal T nucleotide was substituted with Inosine (I) nucleotide in the dye-labelled forward primers in Miniplex 2. For D13S317, as no T nucleotide was present in the primer sequence, an ATTI sequence was attached to the 5' end of the primer. The positions of the U and I nucleotides subsituition are shown in Table 3 and highlighted in bold. As VIC™, NED™ and PET™ are propreitry dyes of ABI and the required U and I nucleotide modifications was not performed by ABI, alternate dyes with similar emission characteristics to the ABI dyes were selected (Table 3). As such, Yakima Yellow for VIC™, ATTO550 for NED™, ATTO565 for PET™ was selected, with the required internal modification of the T nucleotide with etiher U or I nucleotide (EuroGentec, Seraing, Belgium). Primer concentrations were identical to the concenrations used in Miniplex 1 and Miniplex 2. PCR components and thermal cycling parameters are identical to

Miniplex 1 and Miniplex 2 with the only adjustment made by adding both Minplex 1 and Miniplex 2, using 1.5 µl each into one PCR of 15 µl volume.

After PCR, the PCR products were "clean-up" using the MinElute spin column (Qiagen) and eluted using a 15µl volume with EB buffer. 5µl of PCR products were aliquoted into two 0.2ml PCR tubes. To one PCR tube, 0.5 U of Uracil-Specific Excising Reagent (USER™ , New England Biolabs, Ipswich, MA) was added and incubated for 37°C for 30 min. To the second PCR tube, 2.5 U of Endonuclease V, an inosine cleaving enzyme from *Thermatoga maritima* with 1X reaction buffer (Fermantas, Inc., Hanover, MD) was incubated at 65°C for 15 min. The processed samples were then genotyped on the ABI PRISM® 3100 using the same conditions as described earlier. Alleles were manually assigned by comparing to known reference standards using the Genotyper® v3.7 (Applied Biosystems) software.

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Table 3. Primer sequences and assay concentration used in Miniplex C, which uses both sets of primers from Miniplex 1 and Miniplex 2 with an internal T nucleotide being subsituted with U nucleotide (Miniplex 1) and T nucleotide (Miniplex 2) and are bolded. The reverse unlabelled primers remain unchanged. D13S317 primers from Miniplex 1 is removed in the combined primers mix in Miniplex 3.

Locus	Forward Dye Label	Primer Seq (5' to 3')
SRY	6-FAM	GTATCGACC**U**CGTCGGAAG
Amelogenin	6-FAM	CCTTTGAAGTGG**U**ACCAGAGCA
U D2S1338	6-FAM	TGGAAACAGAAA**U**GGCTTGG
U D21S11	6-FAM	ATTCCCCAAG**U**GAATTGC
DYS392	6-FAM	ACCTACCAA**U**CCCATTCCTT
CSF1PO	Yakima Yellow	ACAGTAACTGCC**U**TCATAGATAG
D7S820	Yakima Yellow	GAACACTTGTCA**U**AGTTTAGAACGAAC
D13S317	Yakima Yellow	ATT**U**GGCAGCCCAAAAAGACAGA
TPOX	Dragonfly Orange	CTTAGGGAACCC**U**CACTGAATG
D18S51	Dragonfly Orange	TGAGTGACAAA**U**TGAGACCTT
D16S539	ATTO 565	ATACAGACAGACAGACAGG**U**G
FGA	ATTO 565	AAATAAAATTAGGCA**U**ATTTACAAGC
TH01	6' FAM	CCTGTTCC**I**CCCTTATTTCCC
D19S433	6' FAM	CCTGGGCAACAGAA**I**AAGAT
D13S317	6' FAM	ATT**I**GGCAGCCCAAAAAGACAGA
D3S1358	Yakima Yellow	CAGAGCAAGACCC**I**GTCTCAT
D2S1776	Yakima Yellow	TGAACACAGA**I**GTTAAGTGTGTATATG
D5S818 F	Dragonfly Orange	GGGTGATTTTCC**I**CTTTGGT
VWA	Dragonfly Orange	GGACAGATGATAAA**I**ACATAGGATGGATGG
D8S1179 F	ATTO 565	TTTGTATTTCATG**I**GTACATTCGTATC
DYS390 F	ATTO 565	CTGCATTTTGG**I**ACCCCATA

RESULTS AND DISCUSSION

1. Primer Set Optimization

In determining the optimal primer concentration, several criteria were taken into consideration. Performance criteria, which included overall peak heights, intercolor, intralocus and intracolour balance were targeted on 500pg of genomic DNA. The purpose of the criteria was to enhance the ability of the multiplex assay to generate full DNA profiles from inhibited, degraded or low-levels of DNA samples. A series of primer concentration sets were tested to determine the best primer concentration. Based on the results of each test, the primer concentrations were adjusted in order to achieve the optimum performance. Figure 3 and Figure 4 depicts a representative, 500 pg amplification using Miniplex 1 and 2, respectively. The best overall peak balance was observed at the primer concentration as described in Table 1 and Table 2.

In designing the multiplex assay, miniSTR primers were selected as these primers produced smaller PCR amplicons and their effectiveness in amplification of degraded DNA was well-studied (Chung *et al*. 2004). The exceptions were vWA and D13S317 where published primer sequences from the commercial kit Powerplex 16 (Promega) were selected for both Miniplex assays. The reason for their selection instead of miniSTR primers were due to several discordant alleles found in both vWA and D13S317 (Drábek *et al.* 2004 and Hill *et al.* 2007). In addition, for vWA, the miniSTR amplicon size ranges from 88 to 148 bp (Butler *et al.* 2003) and the Powerplex 16 vWA primers generate amplocons from 123 to 171 bp (Krenke *et al.* 2002) and both primer sets overlaps with D5S818 in Miniplex 2, whose size range is from 106 to 143 bp. Therefore, only the forward Powerplex 16 vWA primer sequence was used while the reverse primer for vWA was re-designed to generate larger PCR amplicons to accommodate both STR markers from overlapping. The use of Powerplex 16

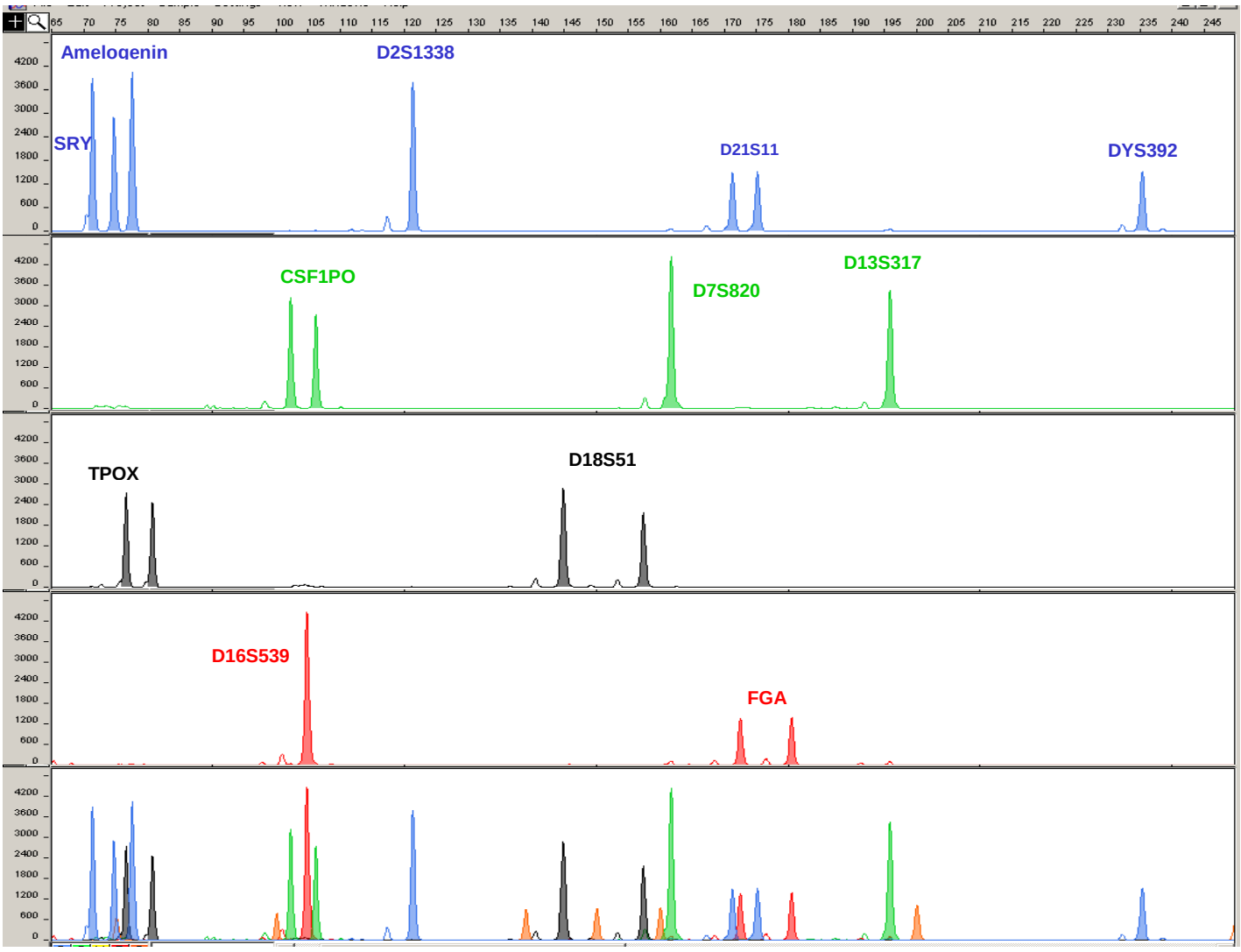


Figure 3. 500 pg of amplification of a genomic sample using Miniplex 1.

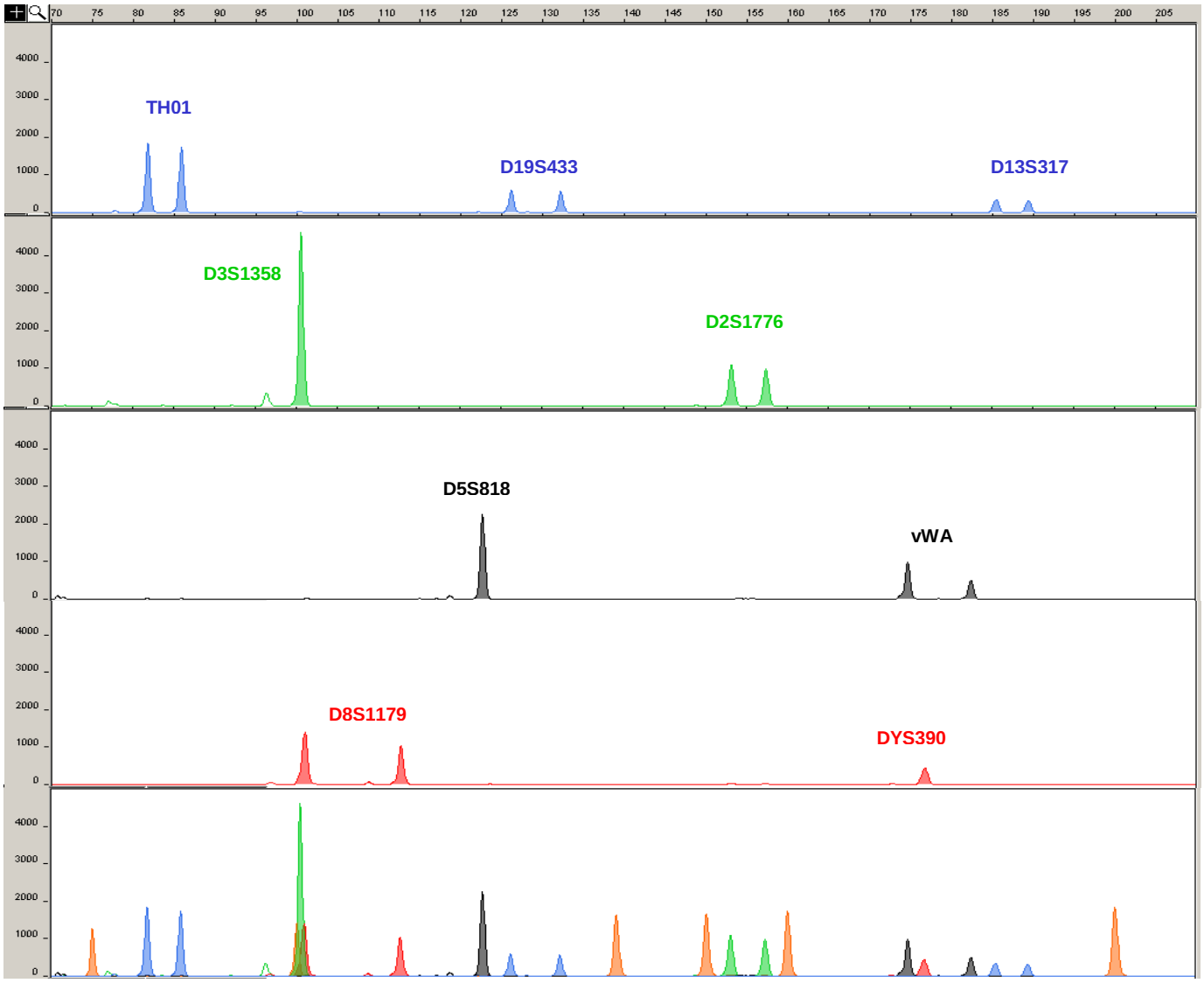


Figure 4. 500 pg of amplification of a genomic sample using Miniplex 2.

D13S317 primers also ensure the amplicons did not overlap with the other STR loci. D13S317 also served as a common STR marker, being present in both Miniplex 1 and 2. This allowed cross-reference of the DNA profiles generated by the two Miniplex assays and served as a check in detecting sample mix-up. D2S1776 was included in Miniplex 2 as a non-CODIS STR locus and is one of 26 new STR markers that has been recently characterized and recommended for use in severely degraded DNA (Hill *et al.* 2008). These new markers were selected as they are unlinked from existing markers from the 13 CODIS loci or at least 50 Mb apart from existing locus used and produced short PCR products in the target region of 50 to 150 bp as these primer sequences were placed directly next to the repeat region unlike some of the CODIS markers. CODIS STRs such as FGA and D7S820 contain partial repeat or monoucleotide repeat stretches that prevent the primers to be designed close to the core repeat region (Butler *et al.* 2002) thus amplicons will need to be larger as the primers are required to be placed away from the repeat stretches. As a result, D2S1776 from the 26 novel MiniSTR markers were selected to be included in our miniplex assays. Our laboratory had recently developed a new multiplex assay, consisting of 8 of these new markers, together with vWA (Lim, unpublished data) to cross-reference with Miniplex 2. Having D2S1776 in Miniplex 2 and the rest 8 MiniSTR markers will allow the laboratory to increase the number of STR markers suitable to genotype degraded DNA samples.

2. PCR Reaction Components and Thermal Cycling Parameters

One of the driving reasons of developing Miniplex 1 and 2 was to overcome PCR inhibition. PCR polymerases such as Phusion Polymerase (Finnzymes, Woburn, MA), OmniTaq and OmniKlentaq (DNA Polymerase Technology); and known PCR enhancers such as Betaine (Sigma Aldrich, St. Louis, MO) and BSA, varying $MgCl_2$ and EDTA

concentrations, PCR additives PEC-1 and PEC-2 (DNA Polymerase Technology) and PCR buffer systems, Gold ST*R buffer (Promega), STRboost® (Biomatric, Inc., San Diego, CA), Rockstart buffer (DNA Polymerase Technology) and EzWay™ Direct PCR Buffer (KomaBiotech, Seoul, Korea) were evaluated either independently or in combinations with one another in a single PCR reaction. These PCR reactions were challenged by increasing concentration of hematin from 0 µM to 150 µM and using 500pg of genomic DNA for amplification and using 30 PCR cycles using Miniplex 1. A summary of this evaluation study is given in Table 4. EzWay™ PCR buffer with OmniTaq had the highest potential in overcoming PCR inhibition. OmniKlentaq was dropped from further evaluation as several non-specific PCR artifacts were observed. In contrast OmniTaq had two distinct non-specific PCR artifacts, approximately 191 and 195 bp in size at the FGA locus. Optimising the PCR annealing and extension temperature in Miniplex 1 can overcome PCR artifacts by increasing the specificity of PCR amplification.

EzWay™ buffer had been used for direct PCR amplification from forensic samples (blood, salvia, sperm, etc.) without any DNA purification step (Park *et al.* 2005, Barbaro *et al.* 2006, Barbaro *et al.* 2008). In Table 4, the buffer when used with AmpliTaq™ Gold (Applied Bioystems, Foster City, CA), could overcome 90 µM of hematin. OmniTaq or Taq22 is a Taq DNA polymerase mutant at codon 708 and had been demonstrated to confer enhanced resistance to various inhibitors of PCR (Kermekchiev *et al.* 2009). Hence from the evaluation study, the combination of EzWay™ buffer and OmniTaq abilities to overcome PCR inhibition when challenged with 150 µM of hematin was not surprising, given both

Table 4. List of tested components for PCR. 1U AmpliTaq Gold (Applied Biosystems) was used when the PCR polymerase is not stated.

Tested components or combinations for PCR	Concentration of hematin when partial DNA profile are observed, Test Range (0 to 150µM)	Remarks
2.5mg of BSA	40µM	
1xPromega ST*R Buffer	40µM	
Addition of Betaine	40µM	
Addition of BSA (2.5mg) with Betaine (0.5M)	40µM	
Phusion Polymerase	-	Amplification failure
3mM of $MgCl_2$	60µM	
4.5mM of $MgCl_2$	50µM	
3µM of $MgCl_2$ with 2.5mg of BSA	60µM	
4.5µM of $MgCl_2$ with 2.5mg of BSA	90µM	
Phusion Polymerase, 3mM MgCl2	-	Amplification failure
Phusion Polymerase, 4.5mM MgCl2	-	Amplification failure
1x EzWay buffer	90µM	
1xEzway buffer, BSA (2.5mg) with 4.5mM $MgCl_2$	20µM	
4.5µM $MgCl_2$ with 2.5mg of BSA	90µM	
Biomatric STRboost	60µM	
OmniKlentaq	-	Non specific amplification products
OmniKlentaq, with 2.5mM EDTA	-	Non specific amplification products
OmniKlentaq with PEC 1	-	Non specific amplification products
OmniKlentaq, PEC 1 with 2.5mM EDTA	-	Non specific amplification products
OmniKlentaq with Ezway buffer	>150µM	Non specific amplification products
OmniKlentaq with 1x Gold ST*R Buffer	-	Amplification failure
OmniKlentaq with PEC2	10µM	Non specific products
OmniKlentaq, 1x Gold ST*R buffer with 2.5mg BSA	-	Amplification failure
1xEzway buffer with 2.5mg BSA	30µM	
OmniKlentaq with Rockstart buffer	-	Amplification failure
OmniTaq	0µM	Partial DNA profile
OmniTaq with ST*R buffer	0µM	incomplete adenlylated PCR products, imbalanced intralocus and interlocus amplification
OmniTaq with EzWay	>150µM	Non-specific amplification products at PCR product at FGA marker

buffer and enzyme were designed to overcome PCR inhibition. The combination enhanced tolerance to PCR inhibitors to those reported for independent studies of either buffer or enzyme assays.

A series of annealing temperatures, which included 55, 57, 58, 59 and 60^{o}C were tested on Miniplex 1 using 500 pg of 007 (Applied Biosystems, Foster City, CA), 9947A (Applied Biosystems, Foster City, CA) and 9948 (Promega Coporation, Madison, WI) control DNA samples. The annealing time for PCR used was 2 minutes instead of 1 minute, even though the amplicons size was under 250 bp. This was done to promote efficiency of primer to DNA template annealing, resulting in more successful DNA polymerization when inhibitory PCR conditions associated typically with forensic samples are encountered. It has been observed that the yield of PCR increased when the annealing time was increased (data not shown). For the annealing temperature tests, PCR artifacts were detected for all annealing temperatures, however at 59 and 60 ^{o}C, the artifacts were below detection levels of 100 RFUs. Generally at increasing annealing temperature, PCR artifacts were reduced at the FGA locus (Fig. 5). 60 ^{o}C were dropped due to allele drop-outs observed at D21S11 and FGA loci. Given the desire to minimise PCR artifacts, maximal specificity and with annealing temperature of 59 ^{o}C having robust PCR amplification with peak heights being balanced, this temperature was determined to be the best annealing temperature for PCR.

A series of extension temperature, which included 63, 67, 70, 72, 74 and 76^{o}C were tested on Miniplex 1. In general, PCR artifacts decreased and PCR products increased with increasing extension temperatures with the exception at 76 ^{o}C, where the PCR products started to decrease instead. It is plausible to conclude that at 76 ^{o}C, the polymerasing activity

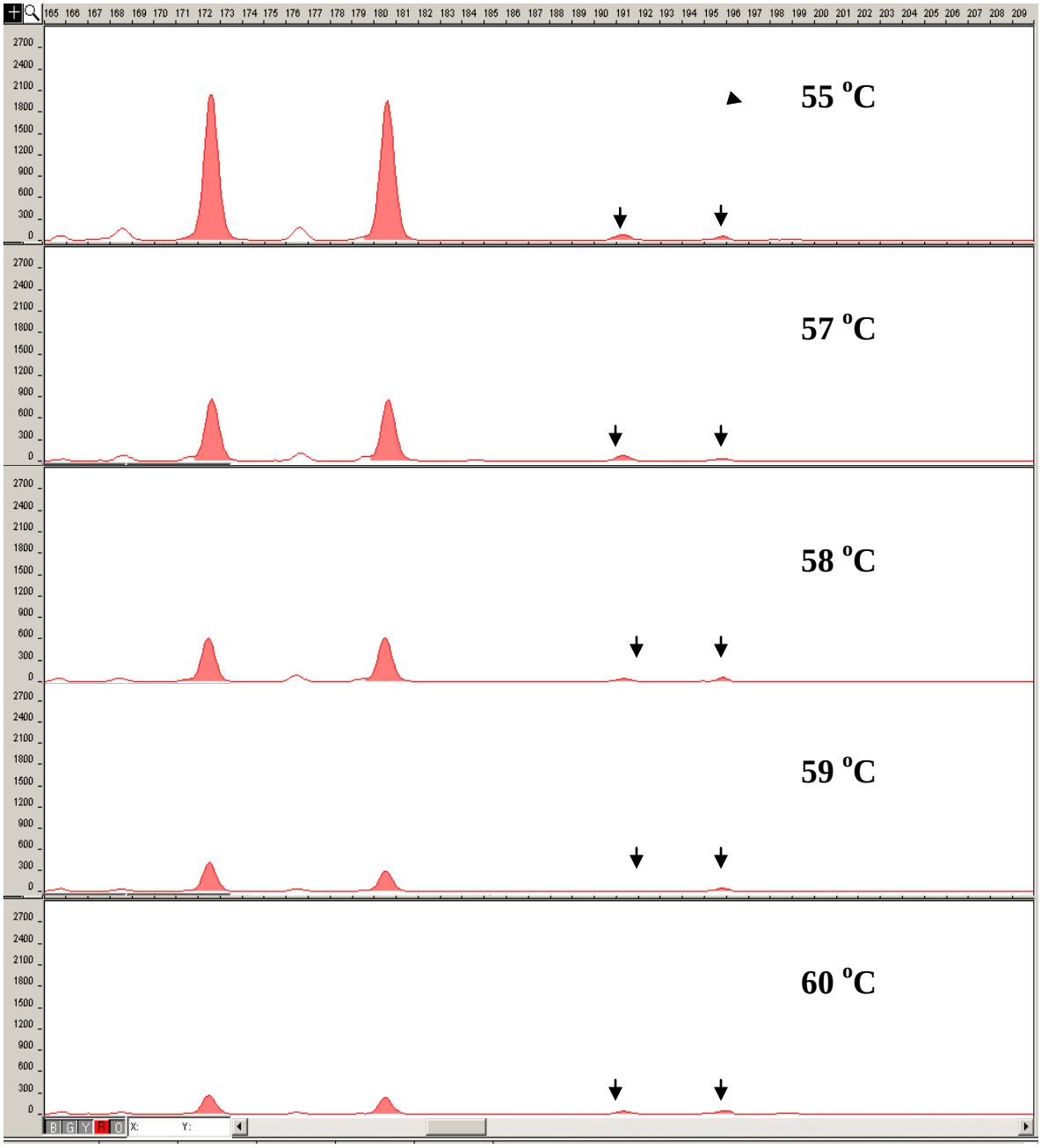


Figure 5. Representative example of the effect of variation in annealing temperature for Miniplex 1 at the FGA locus. Increasing annealing temperature (as indicated in each panel) shows a general decrease of PCR artifacts at 191 and 195 bp are marked by the pointed arrows.

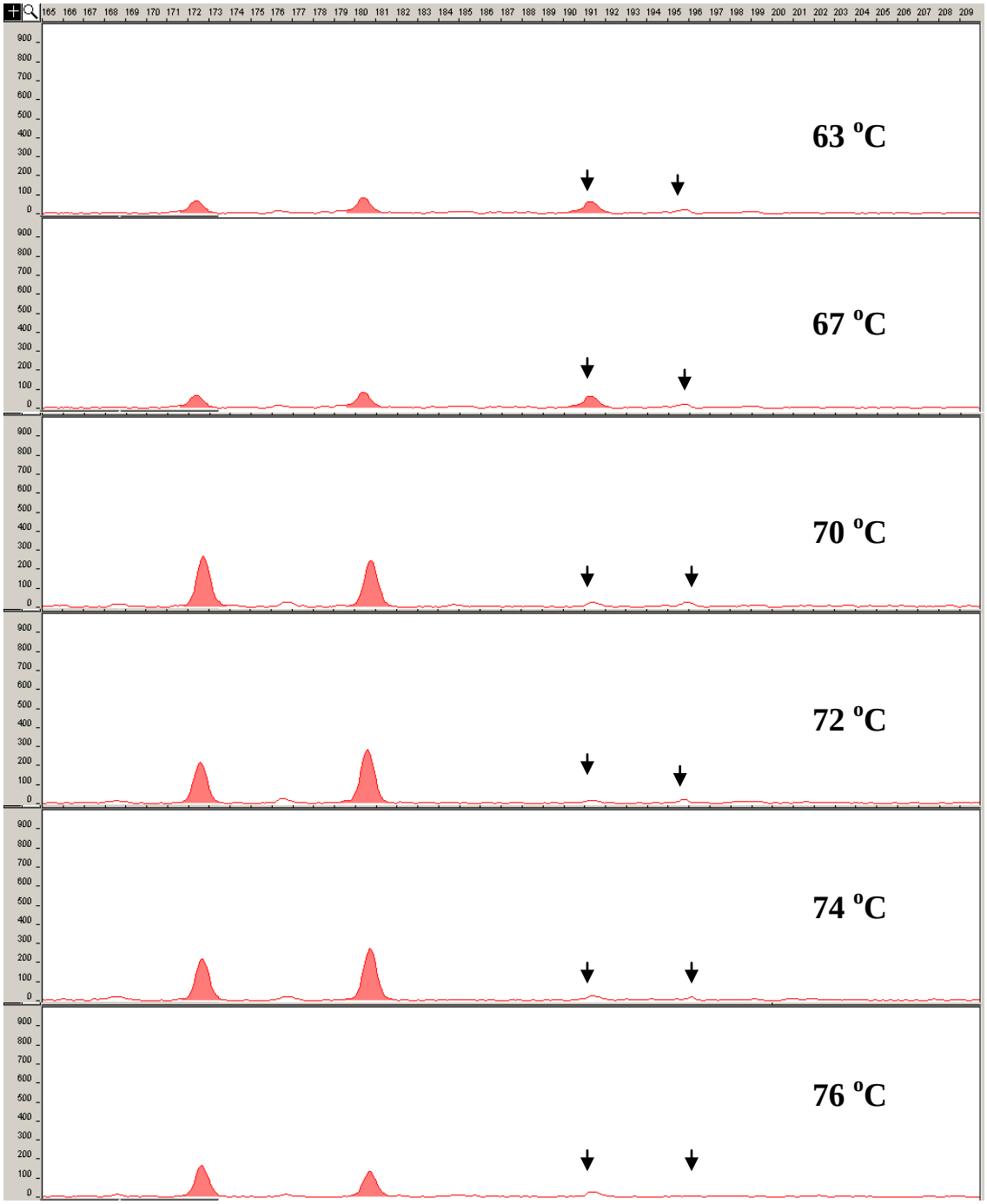


Figure 6. Representative example of the effect of variation in extension temperature for Miniplex 1 at the FGA locus. Increasing extension temperature (as indicated in each panel) shows a general increase of PCR amplicons except at 76°C. PCR artifacts at 191 and 195 bp are marked by the pointed arrows also show a general decrease with increasing extension temperature.

increased specificity of amplification. Complete DNA profile is obtained for the tested annealing temperatures from 55 to 59 ^{o}C, while at 60 ^{o}C, severe interlocus peak imbalance yield is highest and the artifacts were reduced to a minimal. Taken together with annealing of OmniTaq was not optimal, resulting in a decreased PCR product yield. At 74 ^{o}C, PCR increased specificity of amplification. Complete DNA profile was obtained for the tested annealing temperatures from 55 to 59 ^{o}C, while at 60 ^{o}C, severe interlocus peak imbalance yield was highest and the artifacts were reduced to a minimal. Taken together with annealing test, it was determined that the PCR annealing and extension temperature would be fixed at 59 ^{o}C and 74 ^{o}C, respectively. Incremental concentration of $MgCl_2$ i.e. by adding 2 mM, 2.5 mM and 3.5 mM of $MgCl_2$ in the PCR reaction for Miniplex 1 were studied and this resulted in the 191 bp being more pronounced (data not shown) with increasing concentration of $MgCl_2$.

When testing for the PCR components and conditions for Miniplex 2, inefficient amplification and incomplete adenlylation was encountered resulting in split peak morphologies in TH01, D3S3158, D2S1776, D5S818, vWA and D8S1179, which was severely pronounced in D5S818 and vWA (Fig. 7). Wang *et al.* (2008) reported split-peak morphologies in several STR markers in a multiplex assay that was challenged with low pH using acetic acid and increasing the final PCR final extension time to 90 minutes can complete the "+A" addition. In view of this, a final PCR extension time of up to 180 minutes was attempted on Miniplex 2 without any improvement (data not shown). As the split peak morphology had been attributed to low pH (Wang *et al.* 2008), NaOH of varying concentrations are added to the PCR reaction mix on Miniplex 2. This was to determine whether an increase in pH in the PCR reaction could circumvent the incomplete adenlylation and increased the efficiency of PCR amplification. The outcome of the NaOH testing is

shown in Fig. 7. Complete adenlylation of all the STR markers were observed even at the lowest concentration of NaOH of 20 mM. However, at concentrations of 60 mM and above, PCR inhibition was observed, likely a result of the pH being too high for OmniTaq polymerase to function.

To determine whether the amplification conditions for Miniplex 1 was amendable to Miniplex 2, a series of annealing temperatures were tested on Miniplex 2, with other PCR parameters remaining unchanged. The outcome of the testing is shown in Fig. 8. PCR annealing temperature of 58 ^{o}C has the highest and most balanced DNA profile. However an annealing temperature of 59 ^{o}C was chosen instead as the PCR product yield was higher and the conditions would also be identical to that of Miniplex 1, allowing the same PCR protocol to be used for both multiplex assays. Two PCR artifacts at D2S1776 locus were detected at all tested annealing temperatures and are 167 and 171 bp in size. The positions of the PCR artifacts are off the allele positions of known alleles of D2S1776 and would be easily identifiable as PCR artifacts (Fig 8).

For the *Taq* polymerase study, 1U of enzyme was sufficient to amplify 500 pg of DNA at 30 cycles. Higher concentration of enzyme resulted in either over-amplification resulting in PCR artifacts formation or severe inter-locus peak imbalance (Fig. 9). As a result, enzyme concentration of 1U was used for both Miniplex 1 and 2.

Magnesium concentrations were not tested on Miniplex 2. The PCR buffer, EzWay (KomaBiotech) is a pre-formulated PCR buffer with $MgCl_2$ added and has been optimised for PCR amplification (Park *et al.* 2005, Barbaro *et al.* 2008). No further $MgCl_2$ testing was performed and increasing concentration could cause non-specific binding resulting in"allele drop in", which was demonstrated in the $MgCl_2$ titration tests using Miniplex 1.

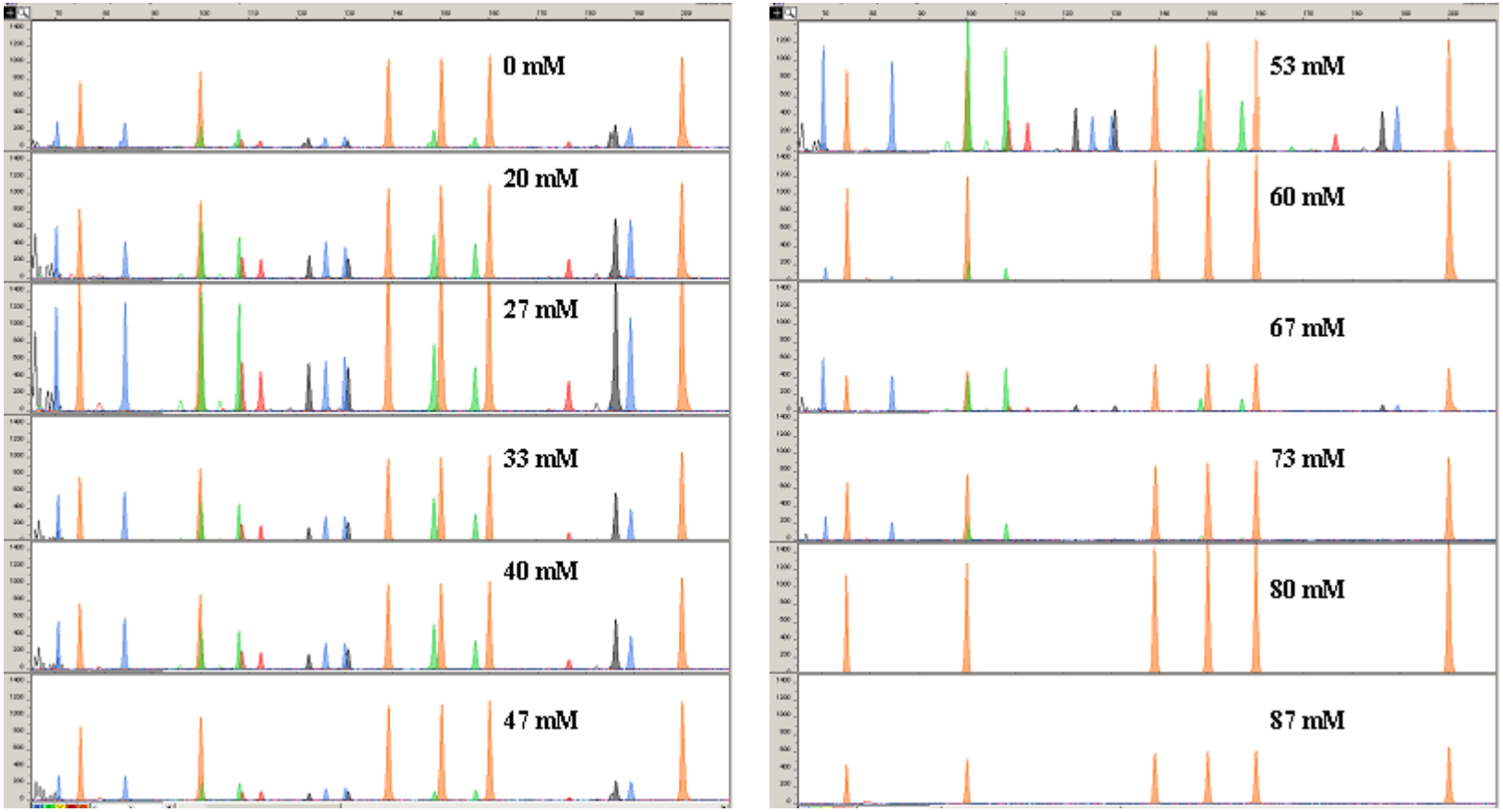


Figure 7. NaOH titration on Miniplex 2. Concentration of NaOH is shown in each panel. In the absence of NaOH, split-peak morphology is observed due to incomplete adenlylation. In the presence of NaOH, complete adenlylation is observed. At 27 mM of NaOH, a balanced and complete DNA profile with the highest amplicon yield is obtained. At NaOH concentrations of 60 mM and above, PCR amplification is inhibited.

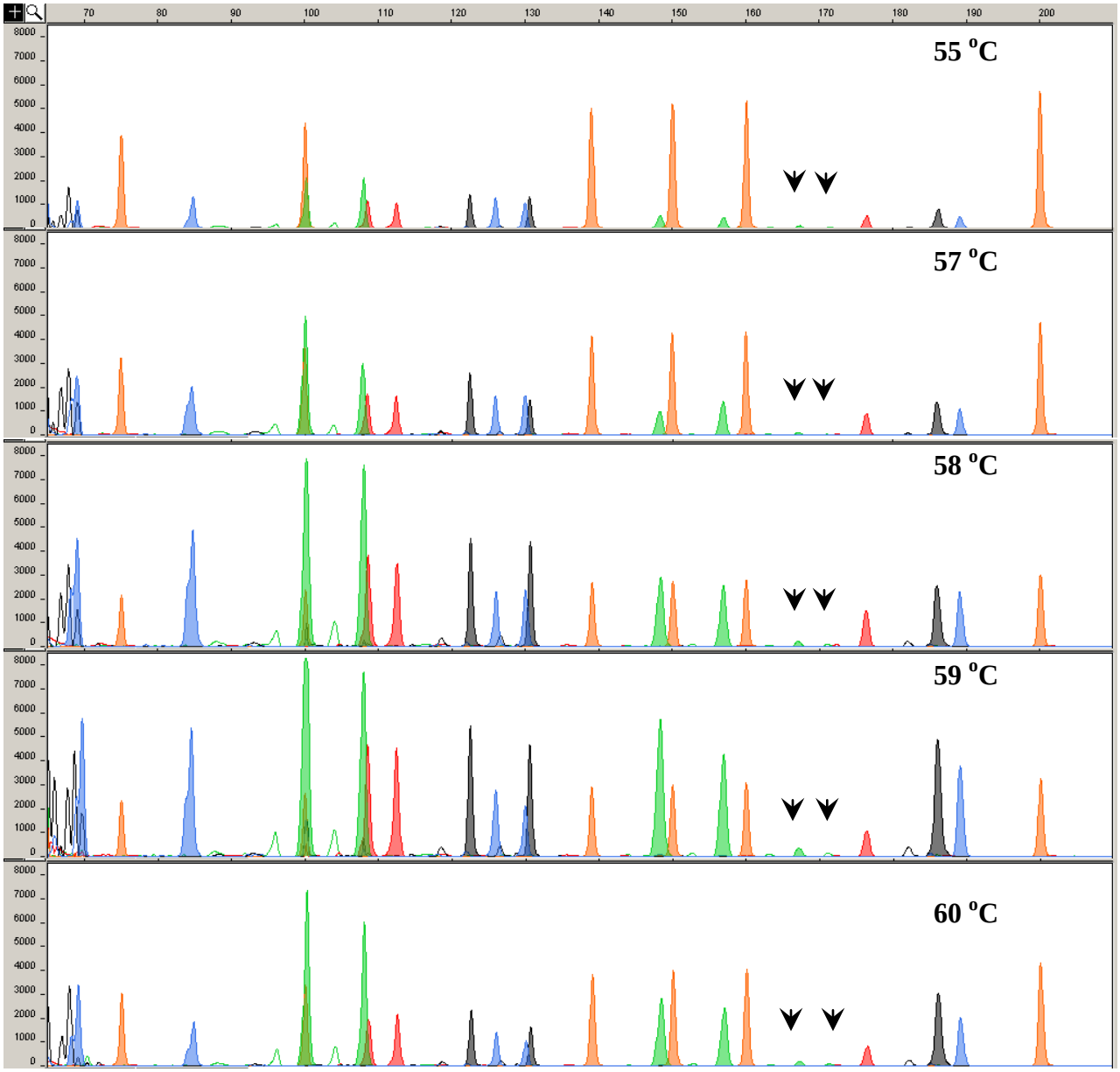
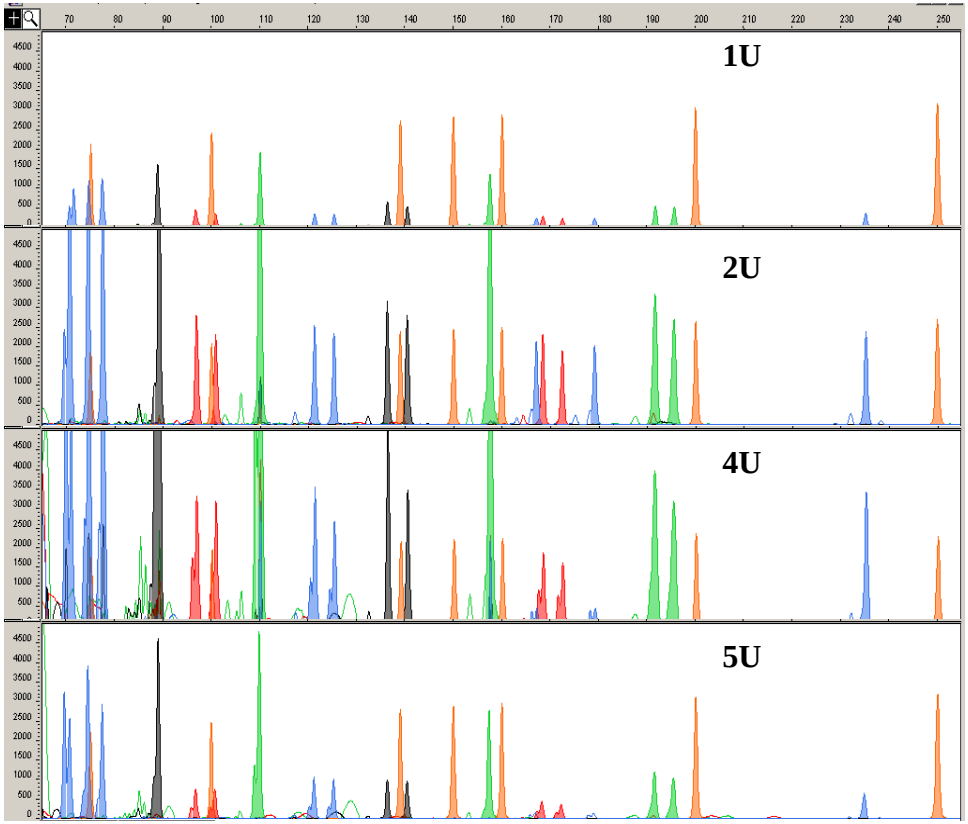


Figure 8. Representative example of the effect of variation in annealing temperature for Miniplex 2 at the FGA locus. Increasing annealing temperature (as indicated in each panel) shows a general increase of PCR amplicons. PCR artifacts at 167 and 171 bp are marked by the pointed arrow which is consistently detectable at all annealing temperatures



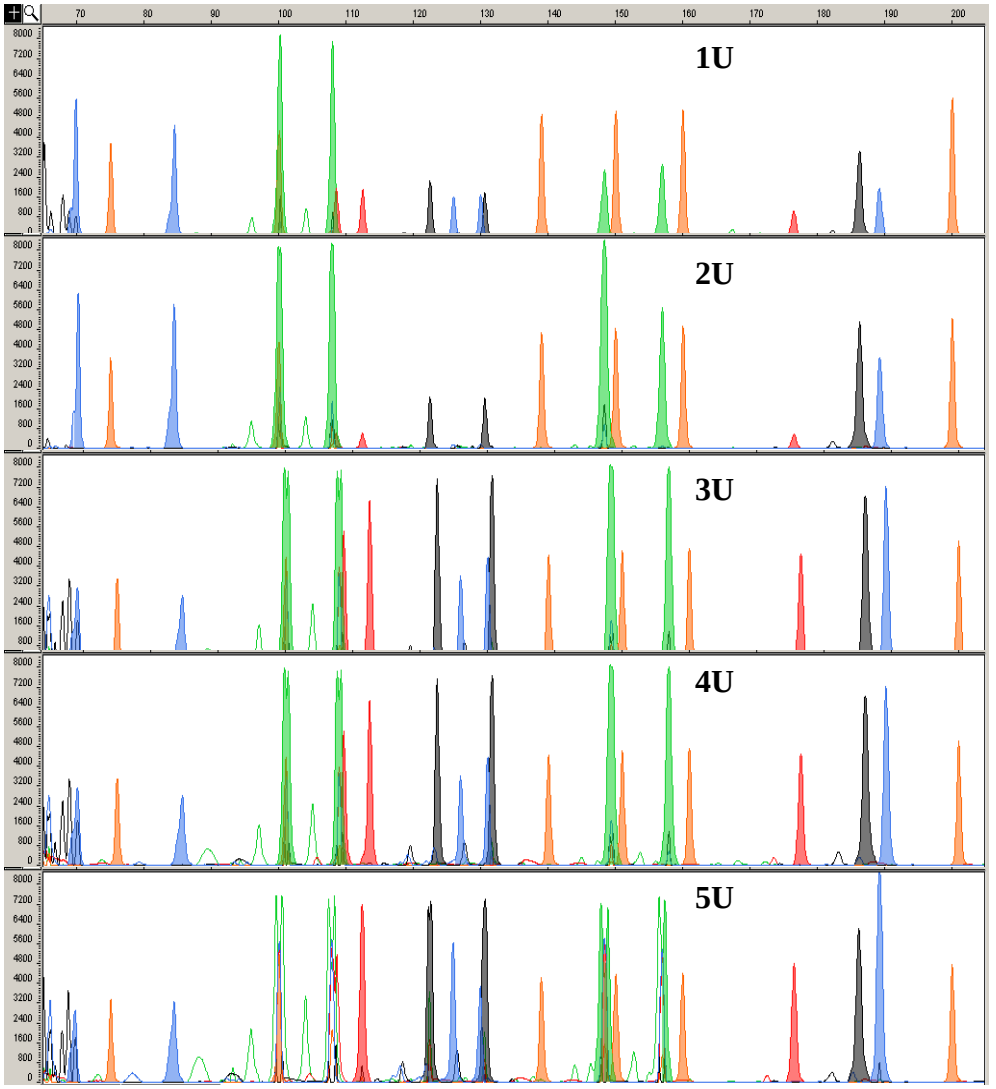


Figure 9. Miniplex 1 (left) and Miniplex 2 (right) titration of OmniTaq DNA polymerase. 500pg/15 µl of DNA template was amplified at 30 cycles and titrated with varying enzyme concentrations as indicated in the panel. Miniplex 1 used R120 male genomic DNA for amplification.

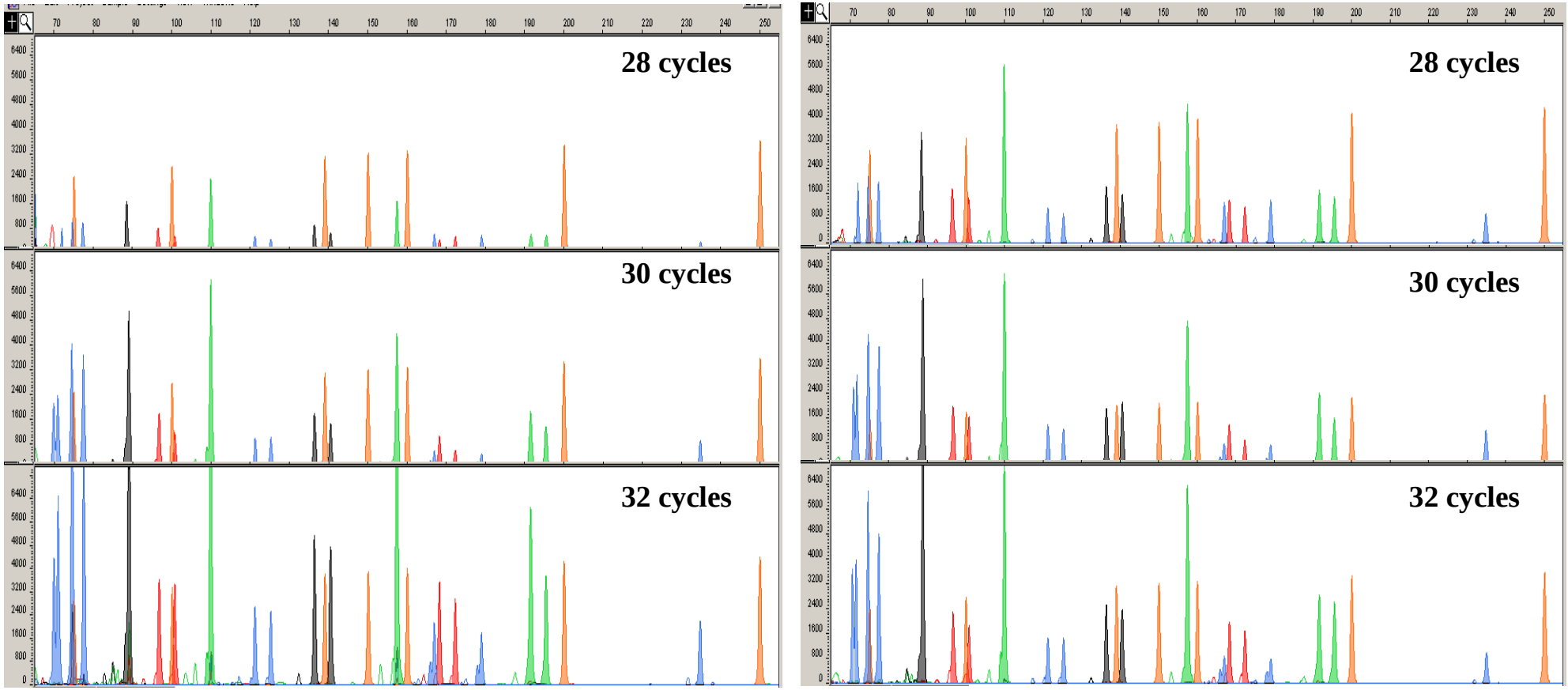


Figure 10. Cycle number study for Miniplex 1. On left, amplifications of 250pg/15µl of DNA at different cycle numbers as indicated in each panel. On right, amplifications of 500pg/15µl at different cycle number. Both miniplex assays used R120 male genomic DNA for amplification.

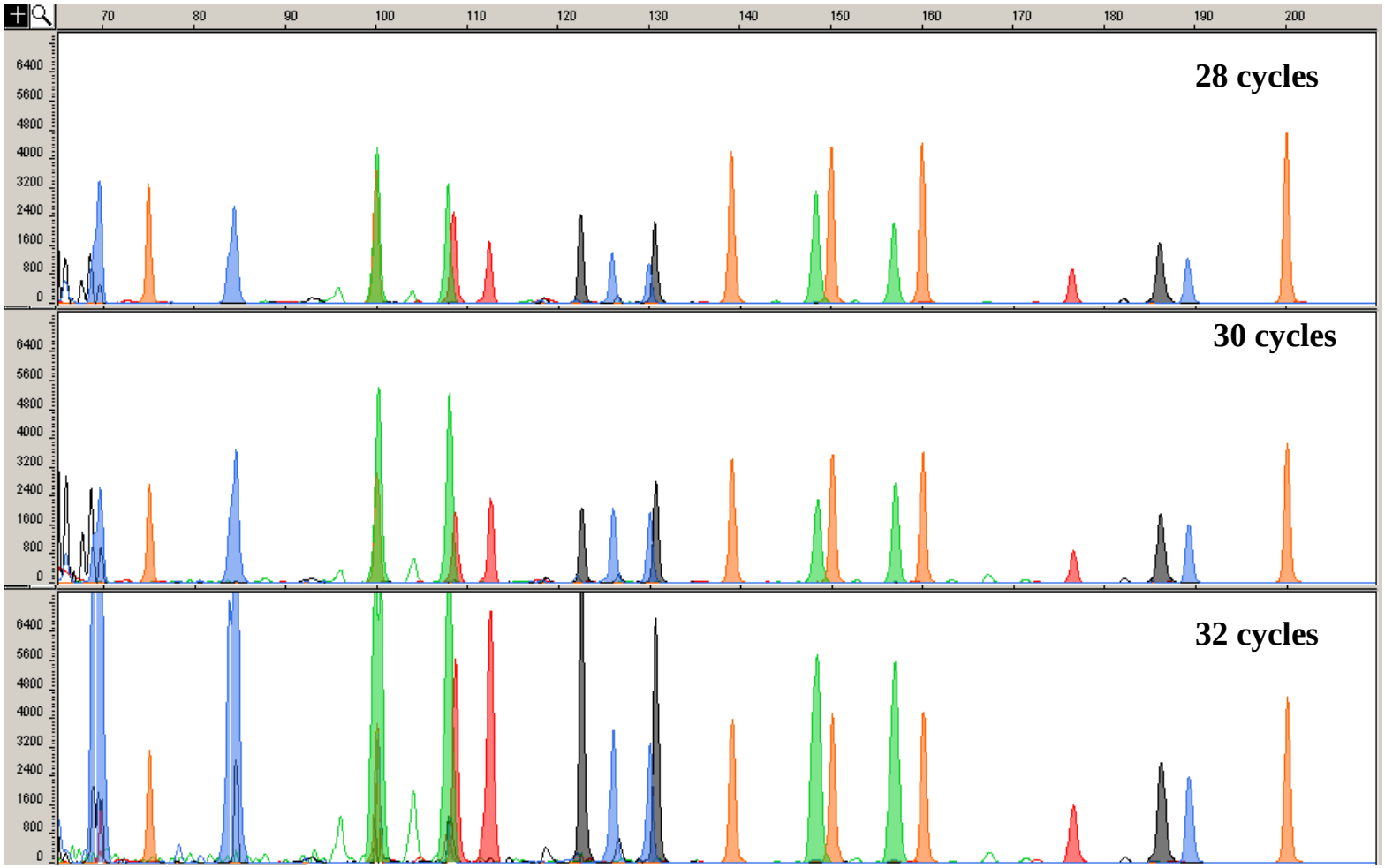


Figure 11. Cycle number study for Miniplex 2. Amplifications were performed using 500pg/15µl of DNA at different cycle numbers as indicated in each panel. 28 and 30 cycles give amplification within the detection limts of ABI 3100 while for 32 cycles, over-amplification is observed for TH01 and D3S1358, which resulted in allele peaks having a split peak morphology.

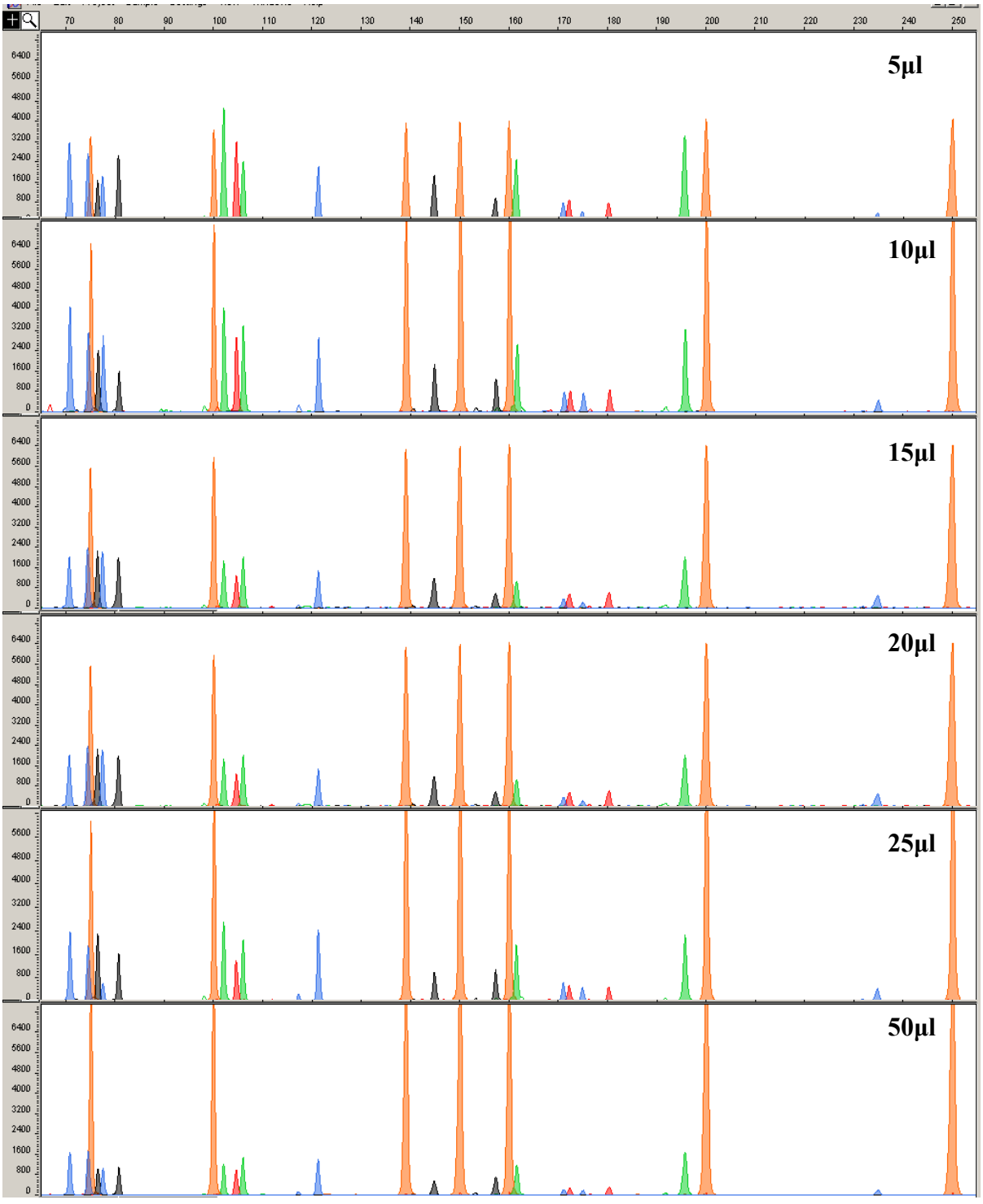


Figure 12. Minplex 1 reaction volume study using 30 PCR cycles and 500 pg of DNA with varying PCR volumes (as shown in each panel). Complete DNA profiles were obtained at all PCR volumes that are studied.

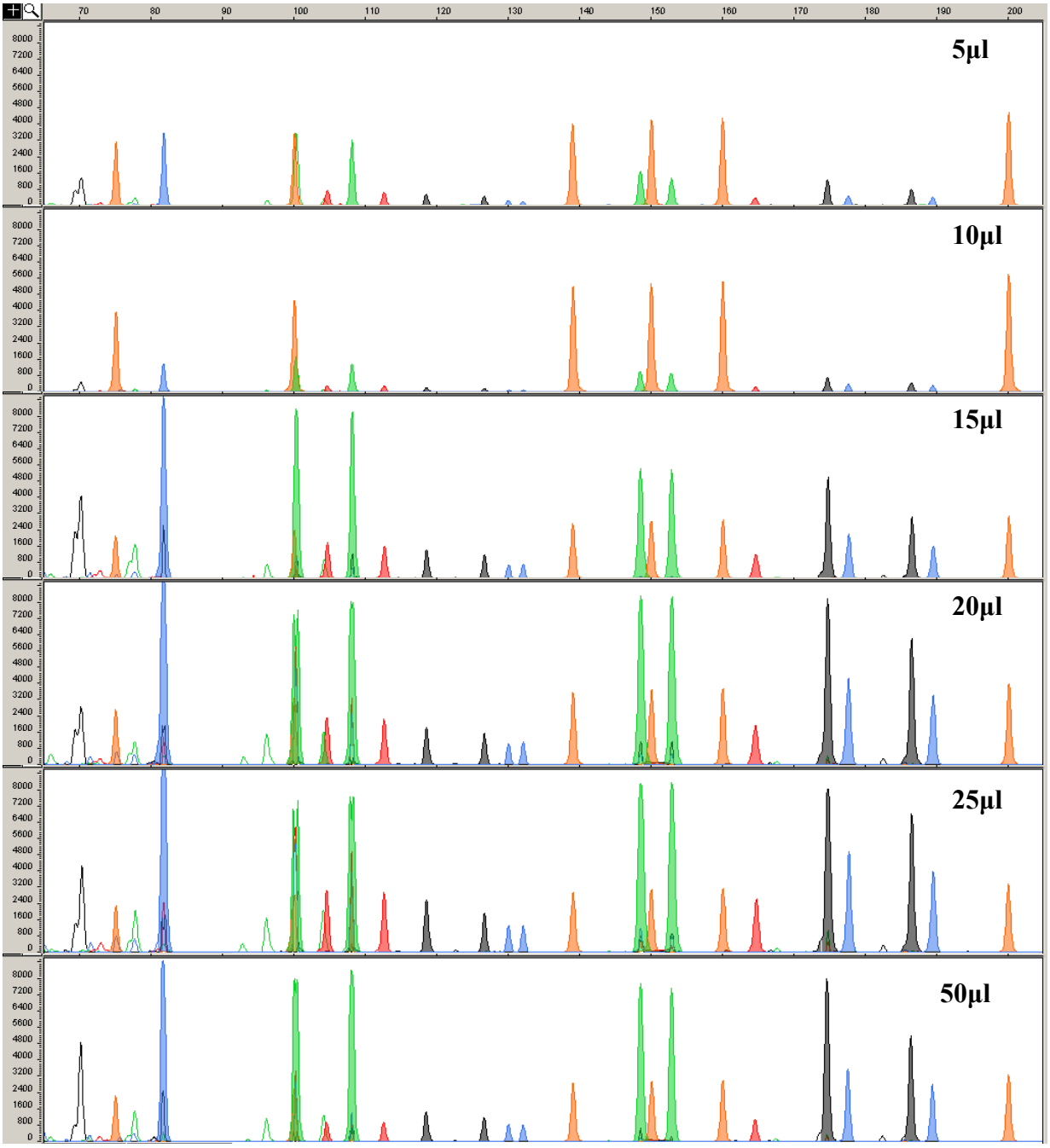


Figure 13. Minplex 2 reaction volume study using 30 PCR cycles and 500 pg of DNA with varying PCR volumes (as shown in each panel). Complete DNA profiles were obtained at all PCR volumes that are studied. R60 male genomic DNA was used in this study.

PCR cycle number studies for Miniplex 1 were conducted using 250pg and 500pg of DNA template over 28, 30 and 32 cycles. For Miniplex 2, 500pg of DNA template over 28, 30 and 32 cycles were tested. Good peak balance was observed for all PCR cycle number and successful amplification was obtained for each of the tested cycles, using Minplex 1 (Fig. 10) and Miniplex 2 (Fig. 11). Overall, increasing the cycle number enhanced the PCR product yield, however lower cycle number could achieve better peak balance. Using 30 cycles for amplifying DNA samples with 500pg is recommended as it achieves the best balance it terms of sensitivity and profile quality.

Reaction volumes of 5-50 µl provided good amplification results for both miniplex assays and produced consistent DNA profiles. It was interesting to note that with Miniplex 1, increasing volumes of PCR reaction resulted in a decrease in signal intensity, a consequent of decreasing amplicons while Miniplex 2, the reverse was true. The result of Miniplex 1 was not surprising, given that the DNA template was being kept constant at 500 pg. Therefore, increasing PCR volume will reduce the sensitivity of amplification process. The difference between the 2 miniplex 2 assays might be due to the addition of NaOH in Miniplex 2, which acted as a PCR enhancer. While the PCR volume has no detrimental effect, to minimize pipetting errors due to small volumes (Cotton *et al.* 2000), the 15 µl PCR volume is chosen.

MiniElute (Qiagen, Inc. Valencia, CA) columns were also used for "PCR clean-up" after amplification. Firstly, to remove 'dye-blobs', an artefact that is due to unattached primer dye labels that co-migrates with the amplicons during genotyping (Butler *et al.* 2003). Secondly, to increase the detection sensitivity of the amplicons during genotyping which has been reported to result in 3 to 8-fold increase in signal intensity (Smith and Ballantyne 2007).

3. Sensitivity, Peak Balance and Stutter

In the sensitivity study, amplification for all loci was obtained for template concentrations as low as 16 pg for Miniplex 1 (n=3) and Miniplex 2 (n=5). Correct genotypes were obtained at concentrations as low as 32 pg for the majority of the samples tested with Miniplex 1 and 2. Allele dropouts started to occur at 125 pg though with longer injection time of 3kv 10s to 3kv 20s would recover the dropout alleles (data not shown). The overall results of the study are presented in Table 5. Correct genotypes were detected at as low as 16 pg, but starting from 125pg, allele dropouts began to occur though limited to the D21S11 in Miniplex 1 and DYS390 in Miniplex 2, gradually affecting other markers as template concentration decreased. Stochiastic amplification resulted when low levels (<100pg) of DNA are used, which can results in either severe allele imbalances or allele droputs. This was observed for DNA tempate concentration of 31 pg and below for both miniplex assays. This was evident by the wide error margins shown in both Fig. 15 and Fig. 17. At 16pg, 16 out of 18 alleles were successfully amplified using Miniplex 1 and for Miniplex 2 14 out of 15 alleles were observed, indicating the high sensitivity of both miniplex assays. Based on these data, template input of 250-500 pg was optimal for Miniplex 1 (Fig. 14) and Miniplex 2 (Fig 16). At this template range, Miniplex 1 and Miniplex 2 was able to achieve intra-locus peak balance higher than 50%, and the average peak heights of 2500 and 1800 RFUs, respectively, which is above our laboratory detection threshold of 100 RFU set for our ABI 3100.

Stutter is a biological artefact, which occurs during PCR due to slippage of the DNA polymerase enzyme (Klintschar and Wiegand, 2003 and Gibb *et al.* 2009) which results in a peak, one repeat unit shorter than the true allele at a much reduced level. Sttuer percentage is

Table 5. Results from sensitivity and peak balance study:

Kit	Template (pg)	Full amplification (%)	Average RFU	Average Peak Balance (%)
Miniplex 1 (n=3)	1000	100	4103	88
	500	100	3284	78
	250	100	1675	76
	125	67	1201	75
	63	33	791	59
	31	0	344	43
	16	0	192	45
Miniplex 2 (n=5)	1000	100	2834	81
	500	100	2274	81
	250	100	1233	82
	125	80	635	71
	63	60	319	39
	31	40	149	21
	16	0	55	16

Template concentrations, percentage of full amplification (detection of all alleles in all loci), average RFU for all loci, and average peak balance for all loci are shown. Miniplex 2 was amplified using R120 male genomic DNA.

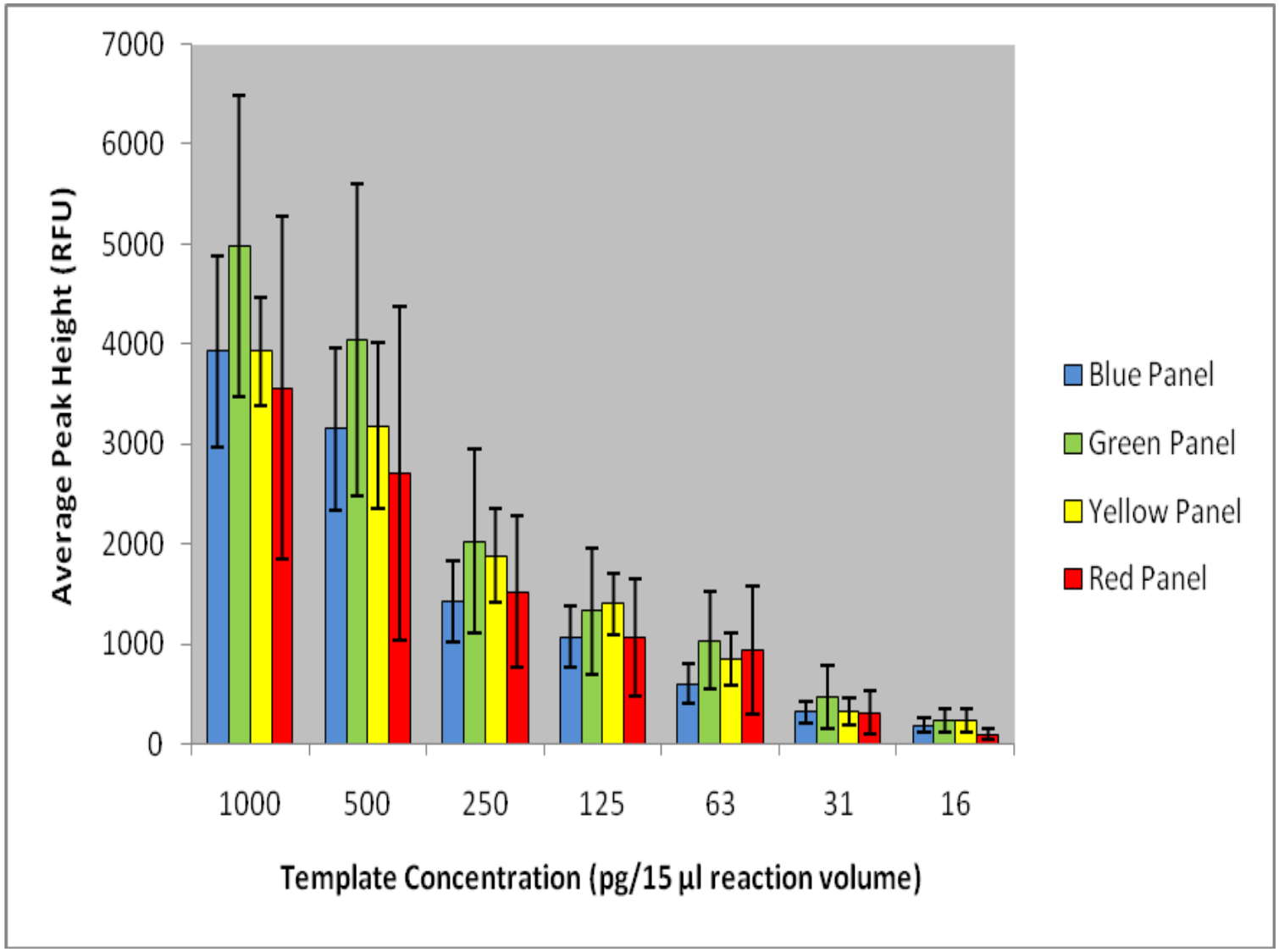


Figure 14. Sensitivity studies for Miniplex 1. The change in fluorescence signal intensity as a function of template concentration is shown. All samples were amplified at 30 cycles. No allele dropout and good signal intensities (> 2500 RFU) were achieved at template concentrations >250 pg / 15µl. Error bars represent ±95% confidence interval from the average peak intensity. Blue panel represents the loci labelled with 6'FAM, SRY, Amelogenin, D2S1338, D21S11, DYS392. Green panel represents the loci labelled with VIC, CSF1PO, D7S820 and D13S317. Yellow panel represents the loci labelled with NED, TPOX and D18S51. Red panel represents the loci marked with PET, D16S539 and FGA.

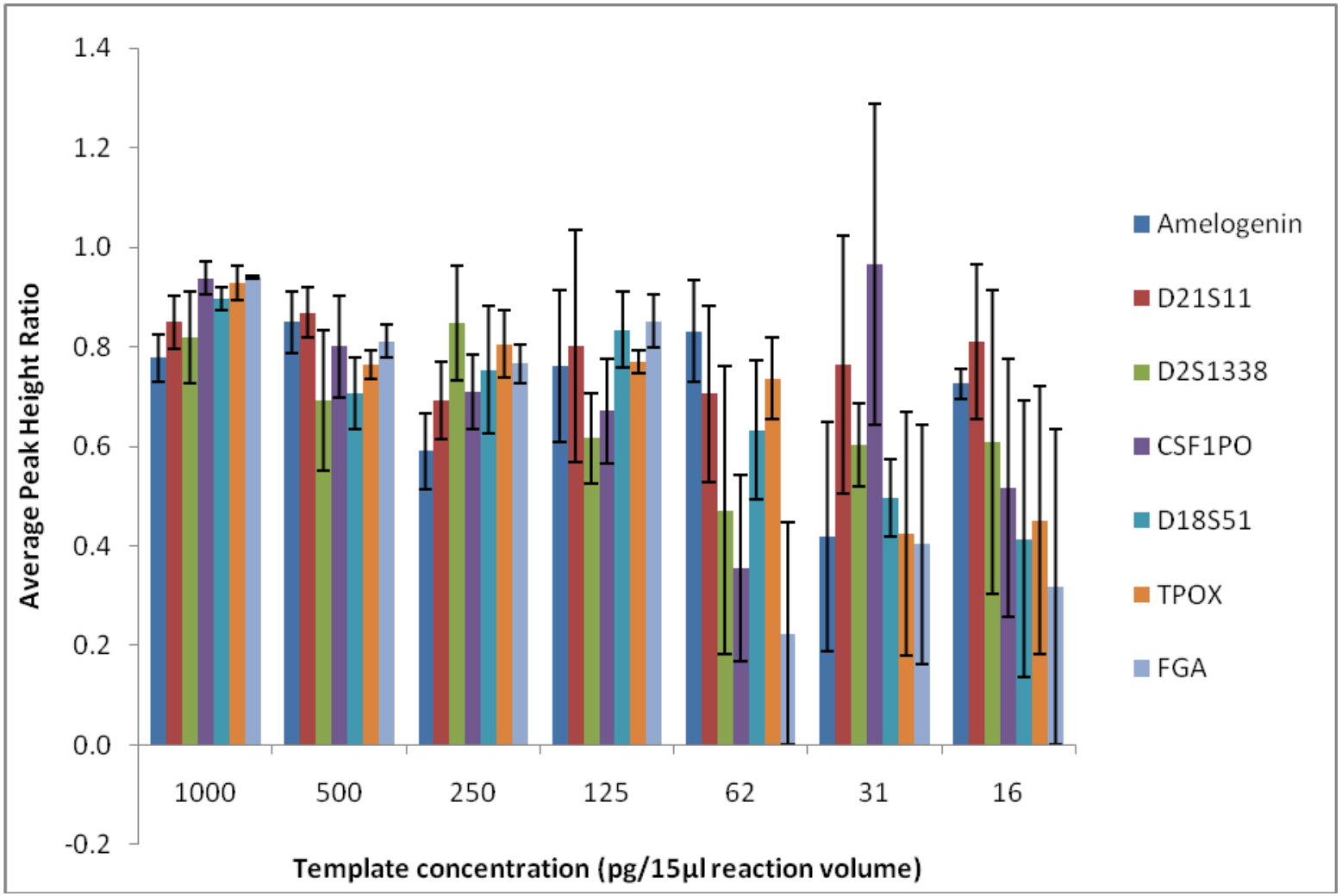


Figure 15. Peak balance ratio for Miniplex 1. The average peak balance ratio for Miniplex 1 is plotted as a function of template concentration. All samples were amplified at 30 cycles. Template concentrations >125 pg／15 µl gave good peak balance ratios (>0.6) for this set at these conditions. Error bars represent ±95% confidence interval from the average peak balance.

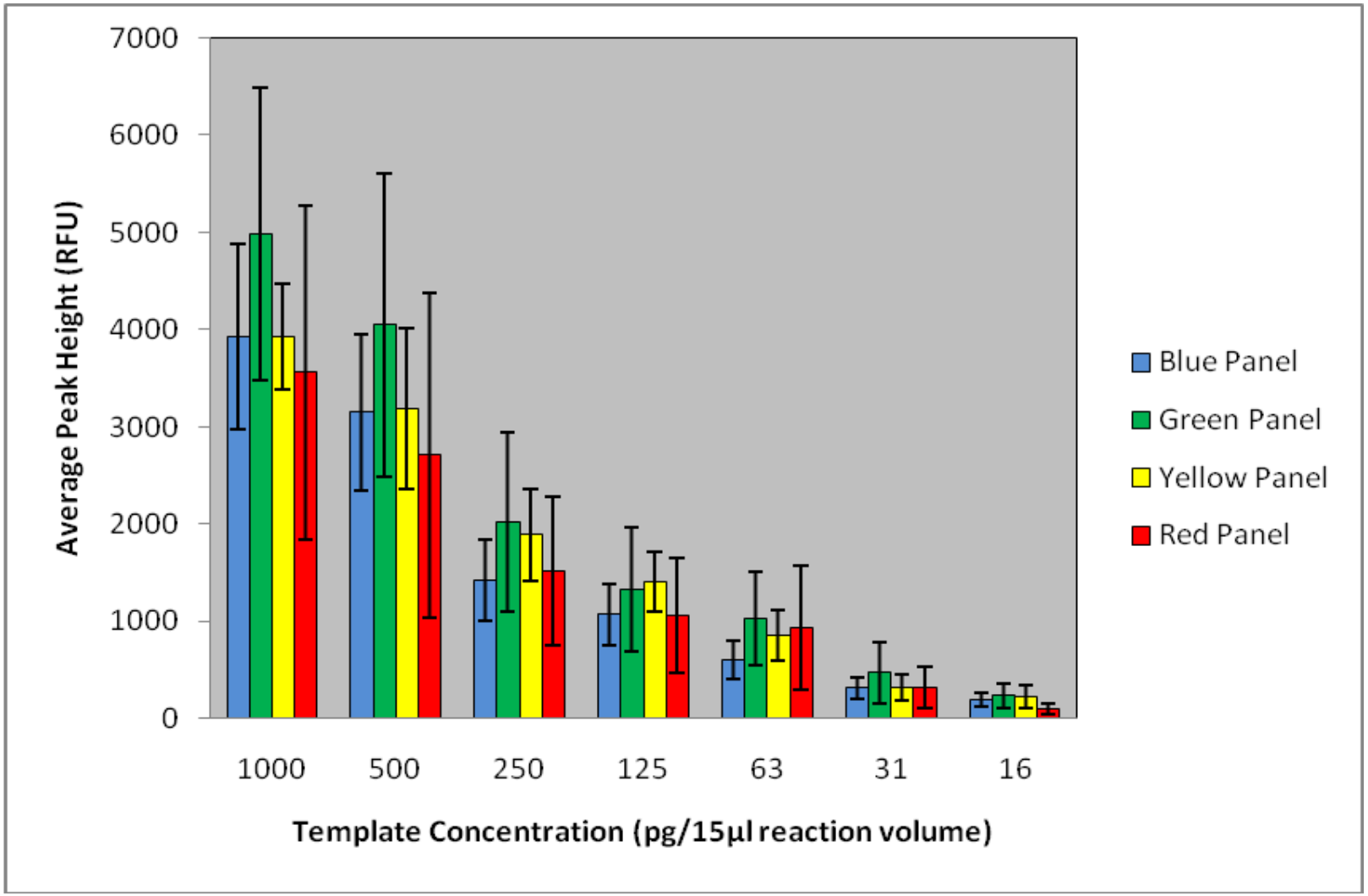


Figure 16. Sensitivity studies for Miniplex 2. The change in fluorescence signal intensity as a function of template concentration is shown. All samples were amplified at 30 cycles. No allele dropout and good signal intensities (> 1500 RFU) were achieved at template concentrations >250 pg / 15µl. Error bars represent ±95% confidence interval from the average peak intensity. Blue panel represents the loci labelled with 6'FAM, TH01, D19S433 and D13S1317. Green panel represents the loci labelled with VIC, D3S1358 and D2S1775. Yellow panel represents the loci labelled with NED, D5S818 and vWA. Red panel represents the loci labelled with PET, D8S1176 and DYS390.

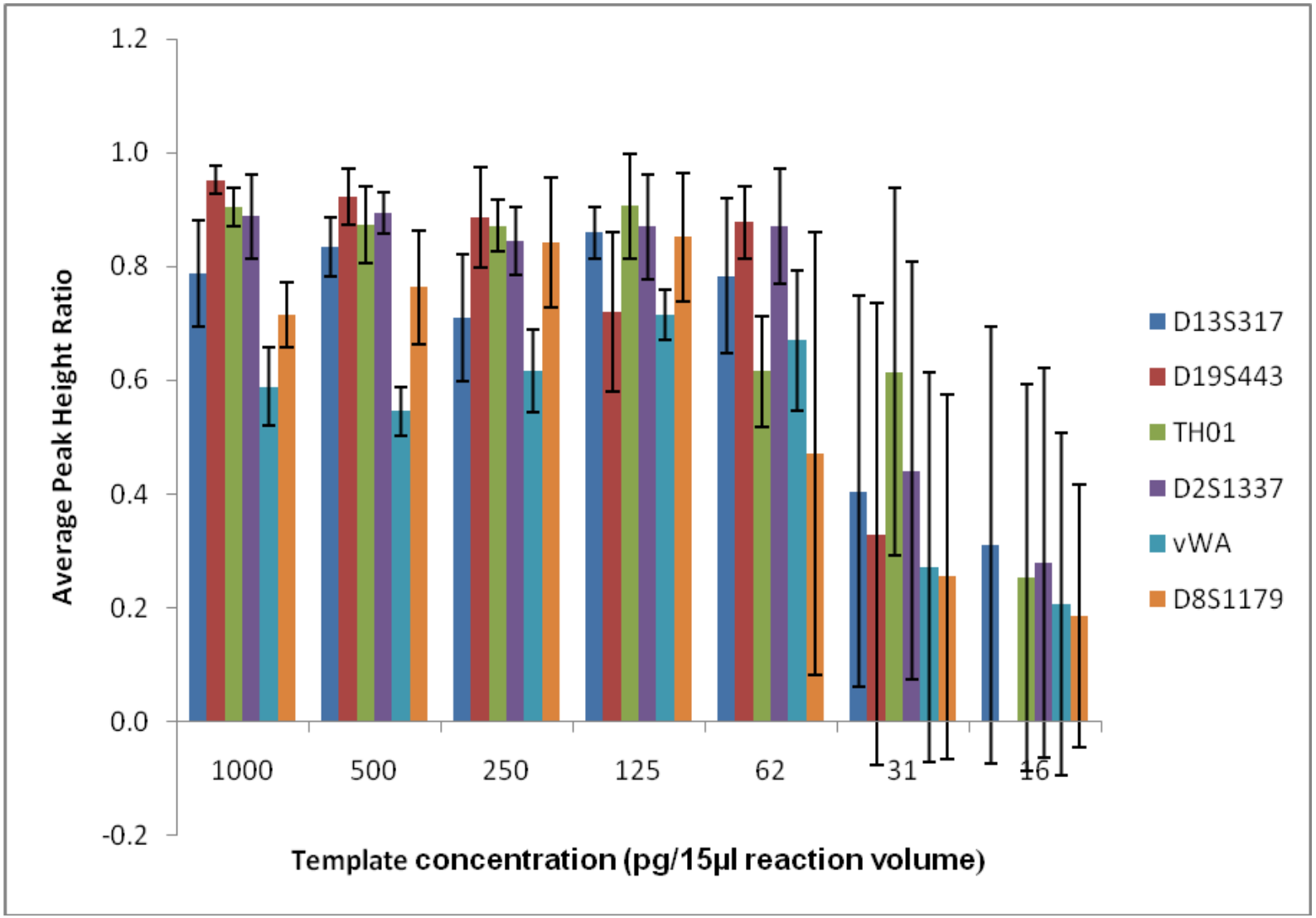


Figure 17. Peak balance ratio for Miniplex 2. The average peak balance ratio for Miniplex 1 is plotted as a function of template concentration. All samples were amplified at 30 cycles. Template concentrations >500 pg／15 µl gave good peak balance ratios (>0.6) for this set at these conditions. Error bars represent ±95% confidence interval from the average peak balance.

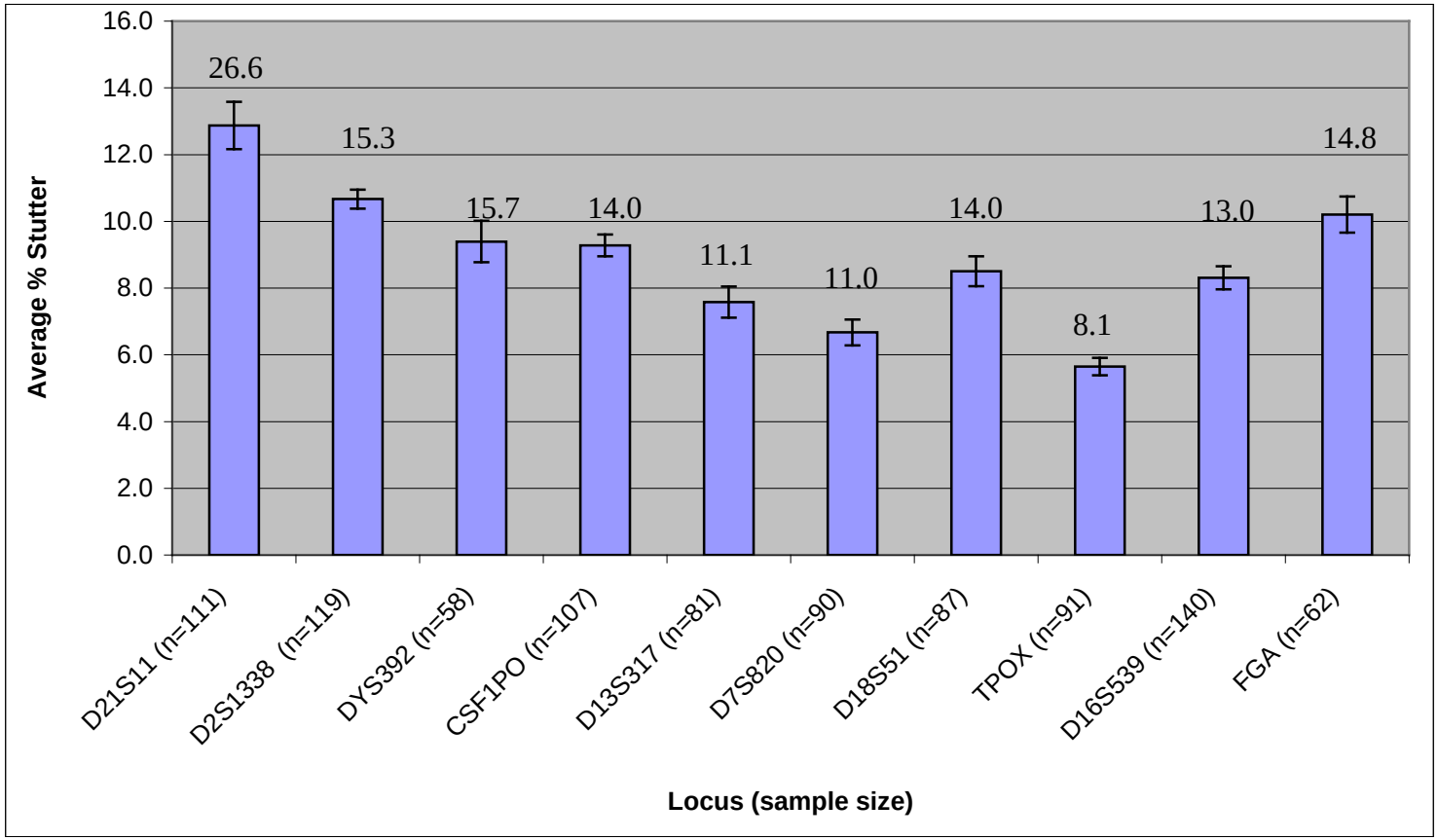


Figure 18. Average stutter calculated for each locus of the Miniplex 1. The sample size (n) indicates the number of samples used to calculate stutter for each locus. Error bars represent ±95% confidence interval from the average stutter value. The highest observed stutter percent for each locus is shown in each column.

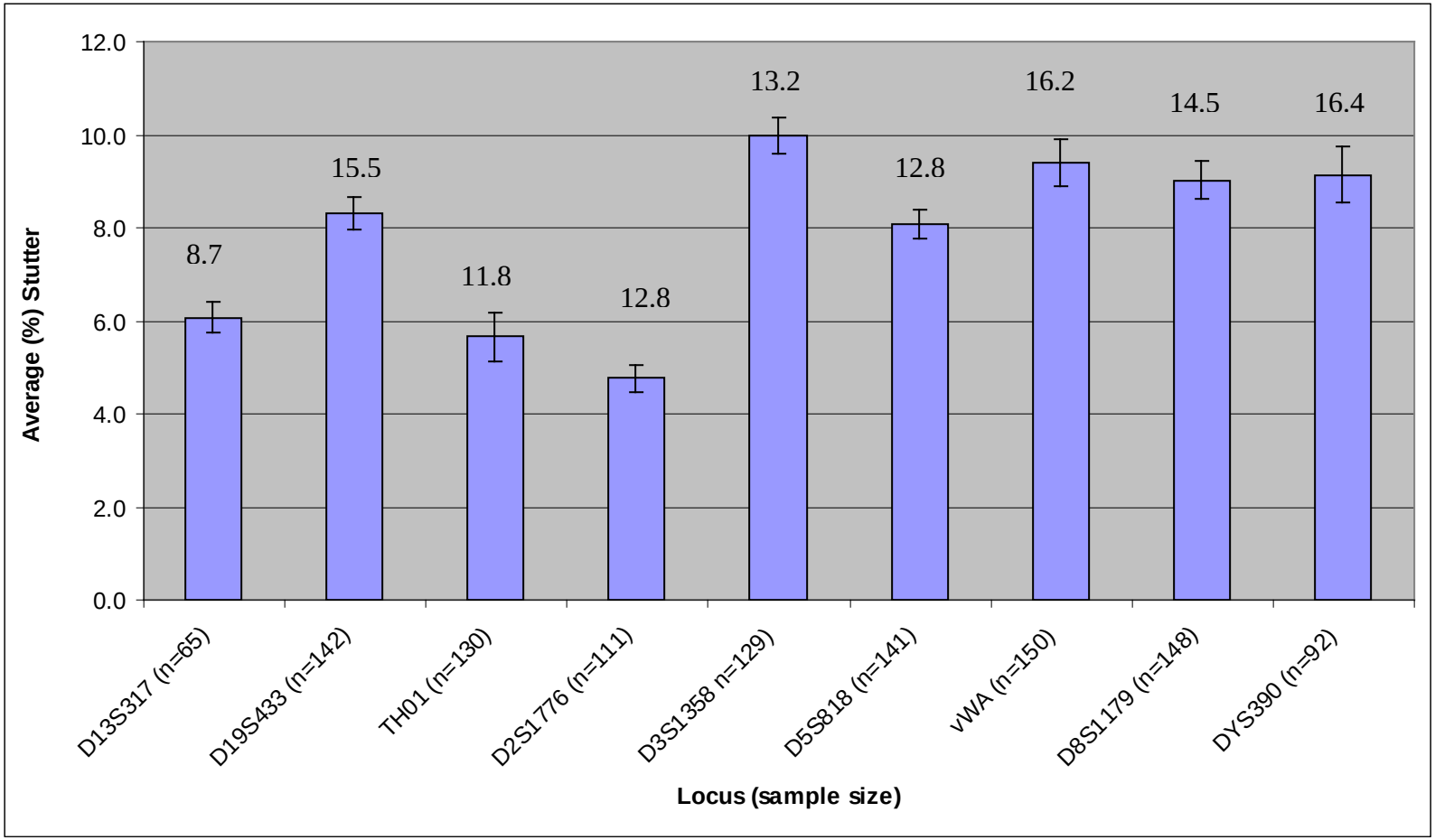


Figure 19. Average stutter calculated for each locus of the Miniplex 2. The sample size (n) indicates the number of samples used to calculate stutter for each locus. Error bars represent ±95% confidence interval from the average stutter value. . The highest observed stutter percent for each locus is shown in each column.

calculated from the ratio of the stutter peak height to that of the true allele. Stutter percentage was less than 17% of all alleles observed except for D21S11, where the highest observed stutter was 26.6%. D2S1776 and TPOX loci were found to have the lowest stutter precentage through its whole panel of alleles (Fig. 18 and Fig. 19). The highest observed stutter for each locus was set as stutter filter threshold as the cut-off level to distinguish true alleles as minor alleles for mixture interpretation from that of stutters. Average stutter percentage increased as alleles became larger or the STR locus had complex or polymorphic STR repeats such as D21S11 and FGA (Frank *et al*. 2001). The amount of stutter appeared higher than Identifiler® and this was likely related to the DNA polymerase processivity, or how rapidly the polymerase copied the template strand. Identifiler® uses Amplitaq Gold while both miniplex assays uses a Taq mutant for amplification.. Stutter products have been shown to increase relative to their corresponding alleles with a slower polymerase (Walsh *et al.* 1996, Meldgaard and Morling 1997), which would mean the processivity of the OmniTaq used for both miniplexes have lower processitivity compared to AmpliTaq Gold used in Identifiler®.

4. Stability

In order to characterise amplification performance in the presence of either inhibited or degraded DNA, stability studies were done, both factors being known to impact PCR efficiency (Adams *et al.* 1991, Akane *et al.* 1994, De Franchis *et al.* 1988, McNally *et al.* 1989, McNally *et al.* 1989). To evaluate the effects of inhibition on amplification, porcine hematin, tannic acid and humic acid, which are common environmental inhibitors, were added in increasing concentrations into PCR. These inhibitors are added unto 500 pg of DNA

template prior to PCR in concentrations varying from 0-500 μM for hematin, 0 to 400 ng/μl of humic acid and 0 to 300 ng/μl of tannic acid.

When challenged with hematin, allele dropouts was first observed with 300 μM of hematin with Miniplex 1 and affects the markers D21S11 and FGA at 1 out of the 5 replicates. For Miniplex 2, alleles begin to dropout at 400μM of hematin. Interestingly for Miniplex 1, with increasing concentration of hematin, the amplifiction appeared to be more robust, evident by the increasing RFUs. Even when the concentration of hematin becomes inhibitory for amplification, the amplified markers still adhere to the trend of increasing peak heights. This effect is less pronounced in Miniplex 2 (Fig. 20 and Table 6). However, for Miniplex 2, it was able to withstand higher concentration of hematin than Miniplex 2 (Fig 21 and Table 7). This could be due to Miniplex 2 having less markers than Miniplex 1, enabling the efficiency of amplification. Another possible reason could be the STR markers found in Miniplex 2 have simple repeats, compared to Miniplex 1 which contains hypervariable complex repeats such as D21S11 and FGA, which were the first loci that experienced dropouts at increasing concentrations of hematin. This effect might be similar to GC-rich DNA template which affects template denaturation during amplification (Frey *et al.* 2008 and Mamedov *et al.* 2008). Both Miniplex 1 and 2 were observed to have increased heterozygote peak imbalance, as reflected by the decreasing peak height balance with increasing hematin concentrations (Table 6). For Miniplex 2, vWA peak height balance is severely affected, as shown in Figure 21. With increasing hematin amounts, it resulted in the smaller allele being amplified preferentially over the larger allele by 4 fold. In commercial kits, locus dropout typically proceeded from larger loci to smaller loci as hematin concentration increased (Collins *et al.* 2004), for Miniplex 1 and 2, this was not as pronouced, as a balanced

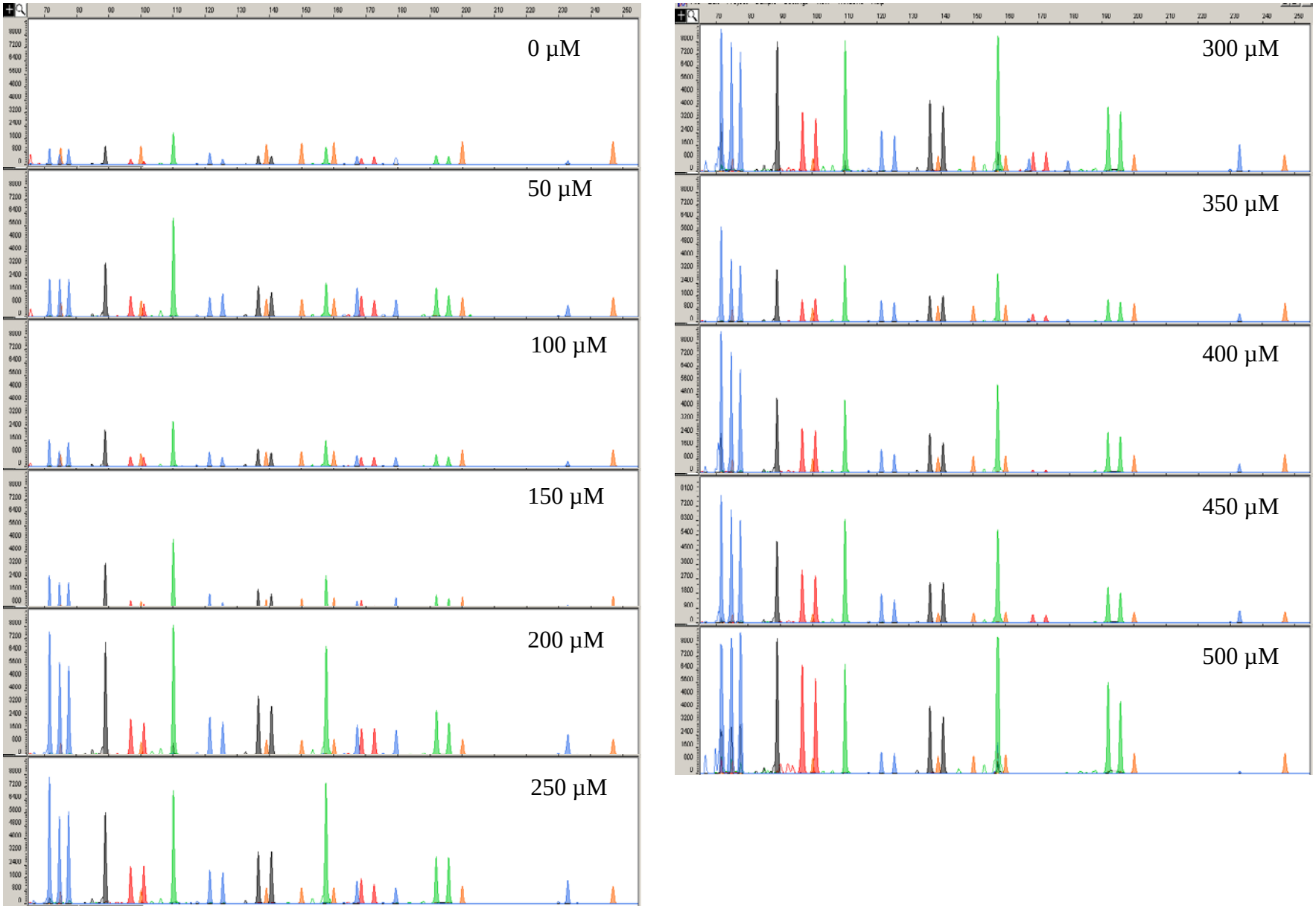


Figure 20. Representative results from stability study of hematin on Miniplex 1. Concentration of hematin shown in each panel. With increasing concentration of hematin, RFUs correspondingly increases though allele dropouts are observed at some loci. Allele dropouts are first observed at 300 µM hematin. However, at 500 µM, 14 out of 19 possible alleles were detectable. R120 male genomic DNA was used in this study.

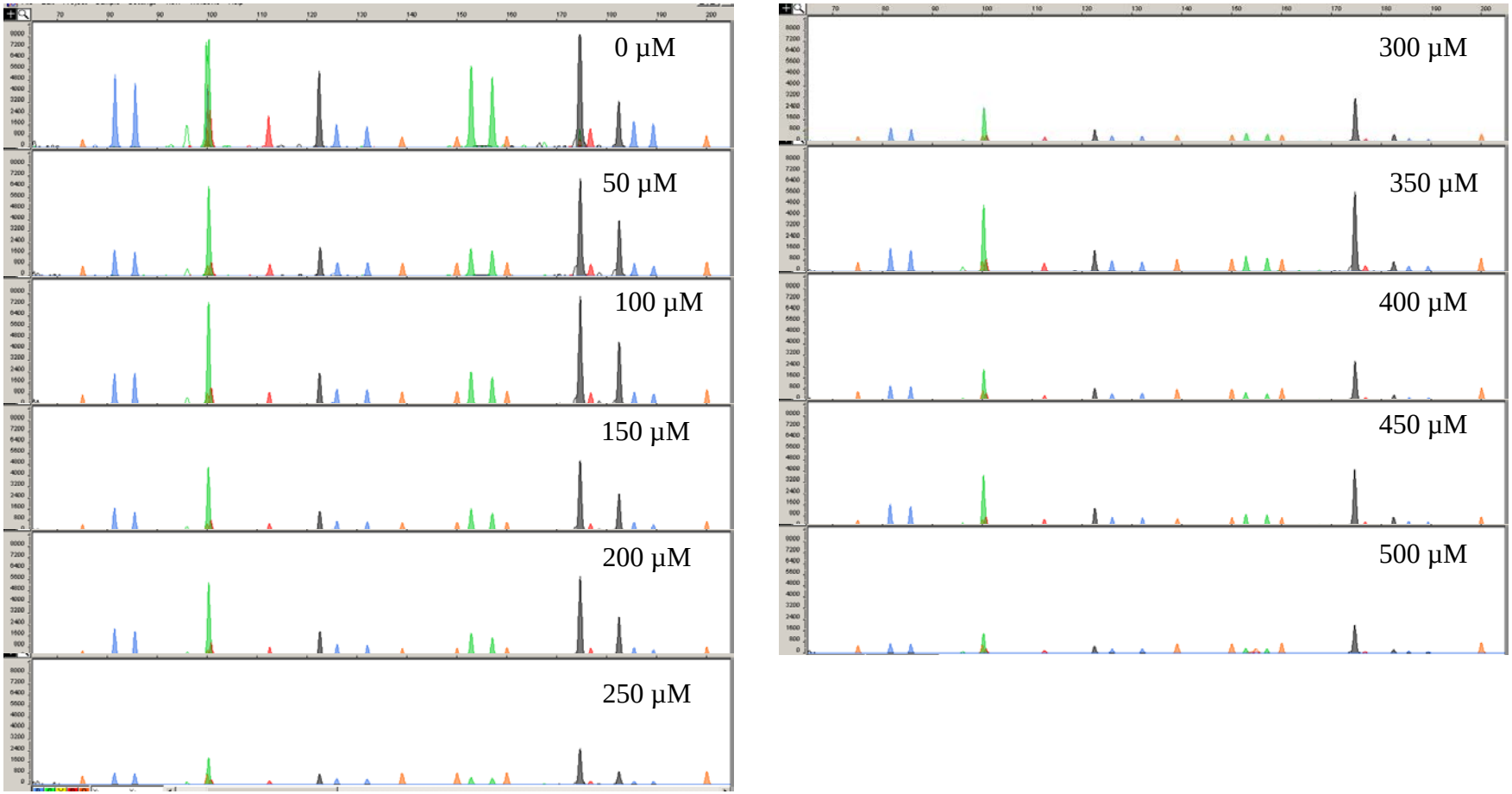


Figure 21. Representative results from stability study of hematin on Miniplex 2. Concentration of hematin shown in each panel. With increasing concentration of hematin, overall peak heights decreases. Allele dropouts are first observed at 400 µM hematin. However, at 500 µM, a complete DNA profile is still obtained. R120 male genomic DNA was used in this study.

Table 6. Results from DNA samples challenged with hematin at increasing concentration.

Kit	Hematin (μM)	Full amplification (%)	Average RFU	Average Peak Balance (%)
Miniplex 1 (n=5)	0	100	1129	79.4
	50	100	2854	80.3
	100	100	4518	85.7
	150	100	2625	77.8
	200	100	4649	86.8
	250	100	3076	85.7
	300	80	4160	78.3
	350	40	2111	66.4
	400	0	4328	55.9
	450	0	4160	57.8
	500	0	2649	36.3
Miniplex 2 (n=5)	0	100	2596	77.3
	50	100	1172	79.1
	100	100	1638	80.6
	150	100	1664	80.2
	200	100	1598	79.1
	250	100	1070	80.2
	300	100	664	78.0
	350	100	815	79.2
	400	80	649	66.4
	450	40	420	28.8
	500	40	163	30.2

Percentage of full amplification (detection of all alleles in all loci), average RFU for all loci, and average peak balance for all loci were studied to study the effects of hematin inhibition on amplification.

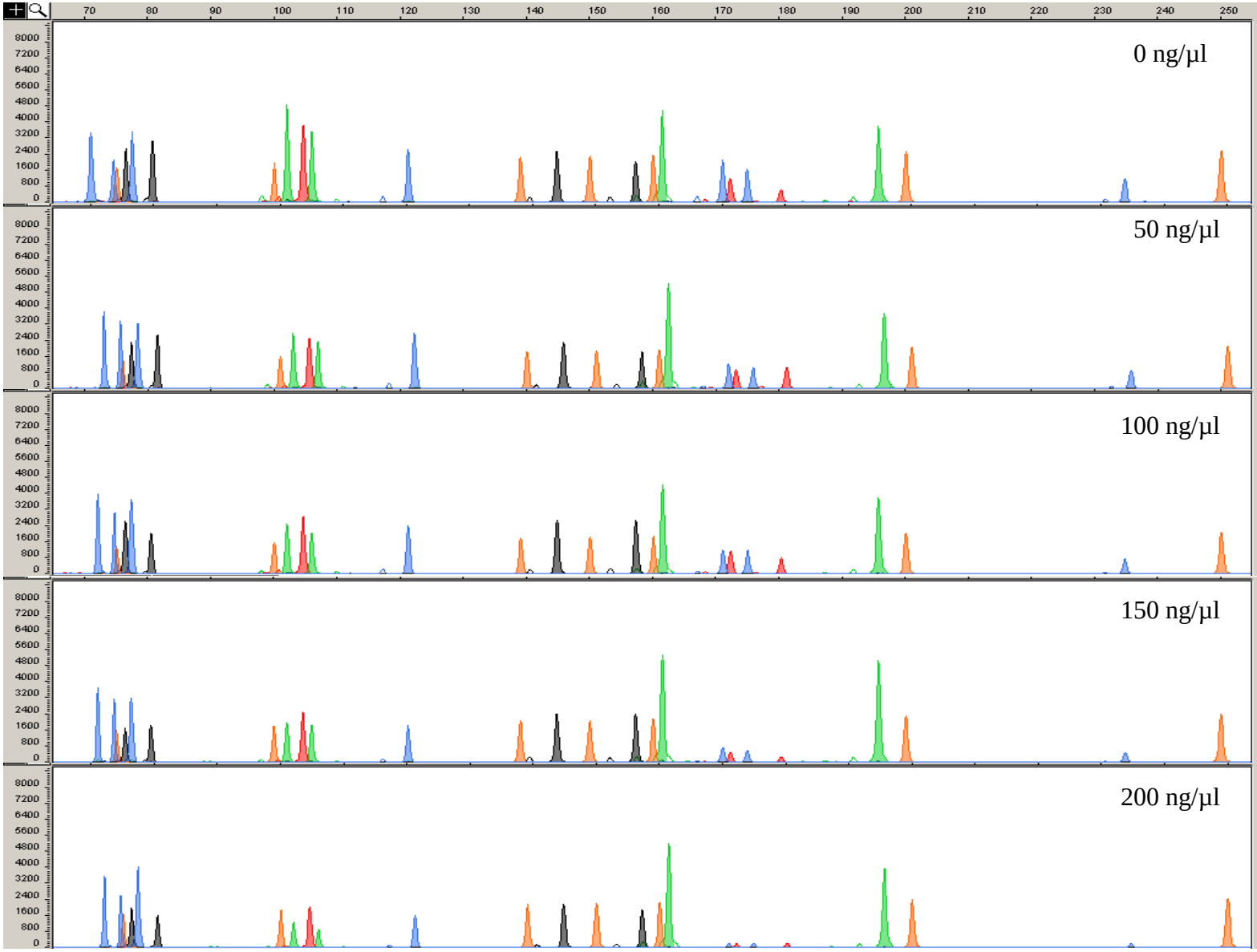


Figure 22. Representative results from stability study of humic acid on Miniplex 1. Concentration of humic acid was shown in each panel. With increasing concentration of humic acid, peak heights of the loci that were amplified remained unchanged though allele dropouts occur at higher concentrations of humic acid. Allele dropouts for one locus, D21S11 were first observed at 150 ng/µl humic acid. However, even at 200 ng/µl humic acid, a complete DNA profile was obtained.

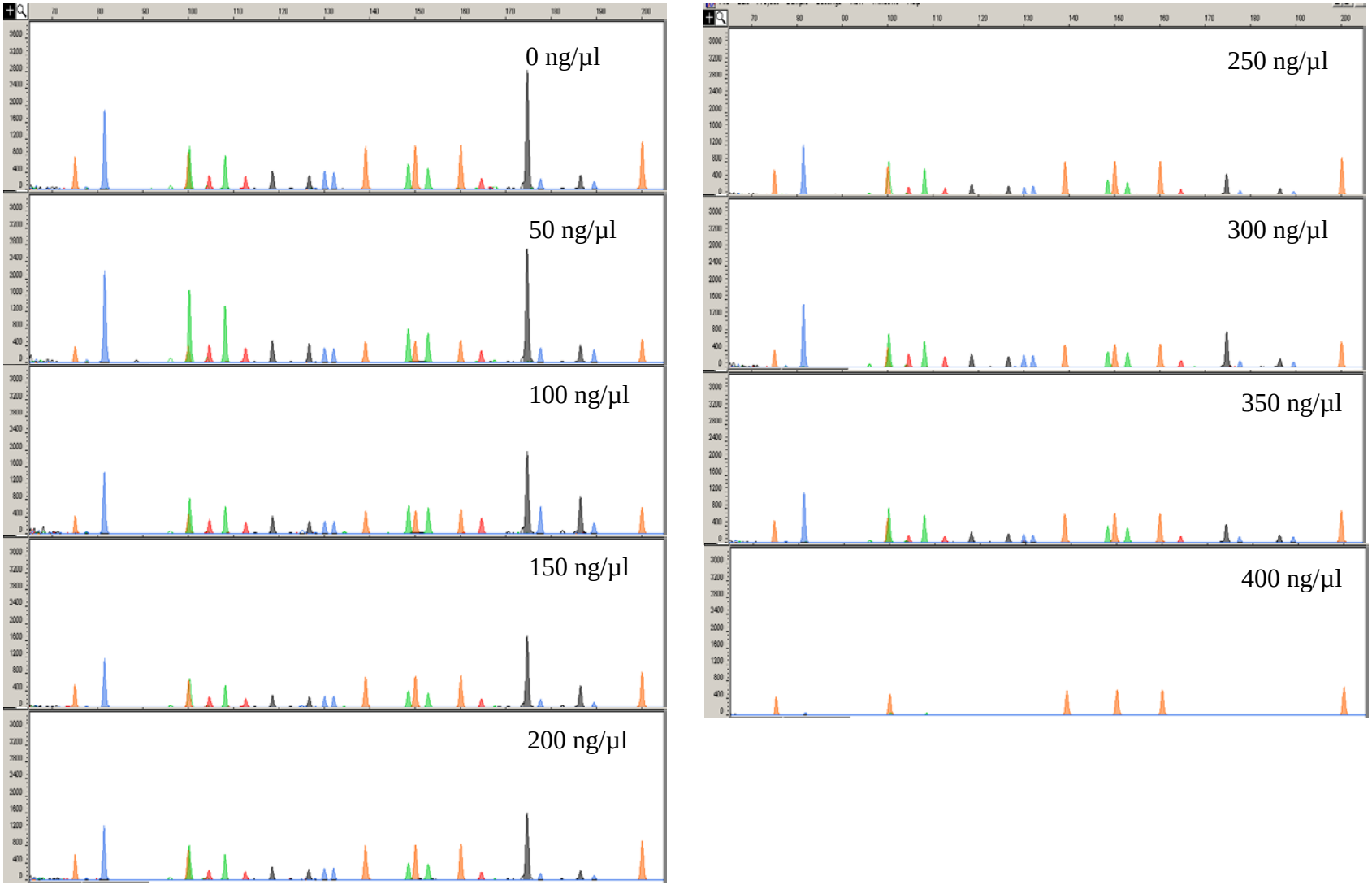


Figure 23. Representative results from stability study of humic acid on Miniplex 2. Concentration of humic acid are shown in each panel. With increasing concentration of humic acid, selected loci are increasing intensity in their peak heights while the rest remains unaffected. Allele dropouts were first observed at 150 ng/µl humic acid. However, even at 350 ng/µl humic acid, a complete DNA profile can be obtained. R60 male genomic DNA was used in this study.

Table 7. Results from DNA samples challenged with increasing concentrations of humic acid.

Kit	Humic Acid (100 ng/μl)	Full amplification (%)	Average RFU	Average Peak Balance (%)
Miniplex 1	0	100	3425	82.3
	75	100	3302	90.3
	100	100	2550	84.5
	150	67	3334	78.9
	200	100	2195	82.9
Miniplex 2	0	100	611	72.0
	50	100	1294	73.6
	100	100	1158	72.2
	150	100	1725	75.6
	200	100	466	75.2
	250	50	297	67.0
	300	100	369	75.6
	350	50	415	49.3
	400	0	98	0

For 0 to 200 ng/µl of humic acid, triplicates for each concentration were tested. For 250 ng/µl to 400 ng/µl of humic acid, duplicates of each humic acid concentration were tested. Percentage of full amplification (detection of all alleles in all loci), average RFU for all loci, and average peak balance for all loci were studied to study the effects of humic acid inhibition on amplification.

reduction is observed. For Minplex 1, a few loci such as SRY, Amelogenin, D16S539, CSF1PO, D7S820, D13S317 showed preferrential amplifcation while the rest of the loci have reduced peak heights

For samples that were challenged with humic acid, the observations were similar to hematin (Fig. 20, Fig. 21 and Table 6) by which increasing concentrations of humic acid resulted in decreased amplification of several loci, especially D21S11 and FGA in Miniplex 1 while for Miniplex 2, a more balanced decrease across all loci is observed. Kermekchiev and co- workers (Kermechiev *et al.* 2009) reported Taq 22 mutant or OmniTaq, which is the DNA polymerase used in the miniplex assays, that humic acid has a stimulatory effect on the mutant DNA polymerase. This stimulatory effect was more prononced in Miniplex 2, where concentrations of between 50 ng/µl to 150 ng/µl of humic acid enhanced PCR, but concentrations above that resulted in PCR inhibition (Fig 21). This could potentially mean that adding 50 ng/µl to 150 ng/µl of humic acid could act as a novel PCR enhancer specific to the mutant OmniTaq polymerase.

For samples that were challenged with tannic acid, the results were surprising. Miniplex 1 was able to withstand inhibition of up to 150 ng/µl of tannic acid, without any of the samples showing allele dropouts and full amplification was still observed at 250 ng/µl of tannic acid (Fig. 24 and Table 8). For Miniplex 2, the presence of tannic acid caused a reduction in amplification efficiency. However, amplification with increasing concentration of tannic acid remains consistent (Fig. 25 and Table 8) without any detriment to PCR except when tannic acid level reached 250 ng/µl, allele dropouts were observed.

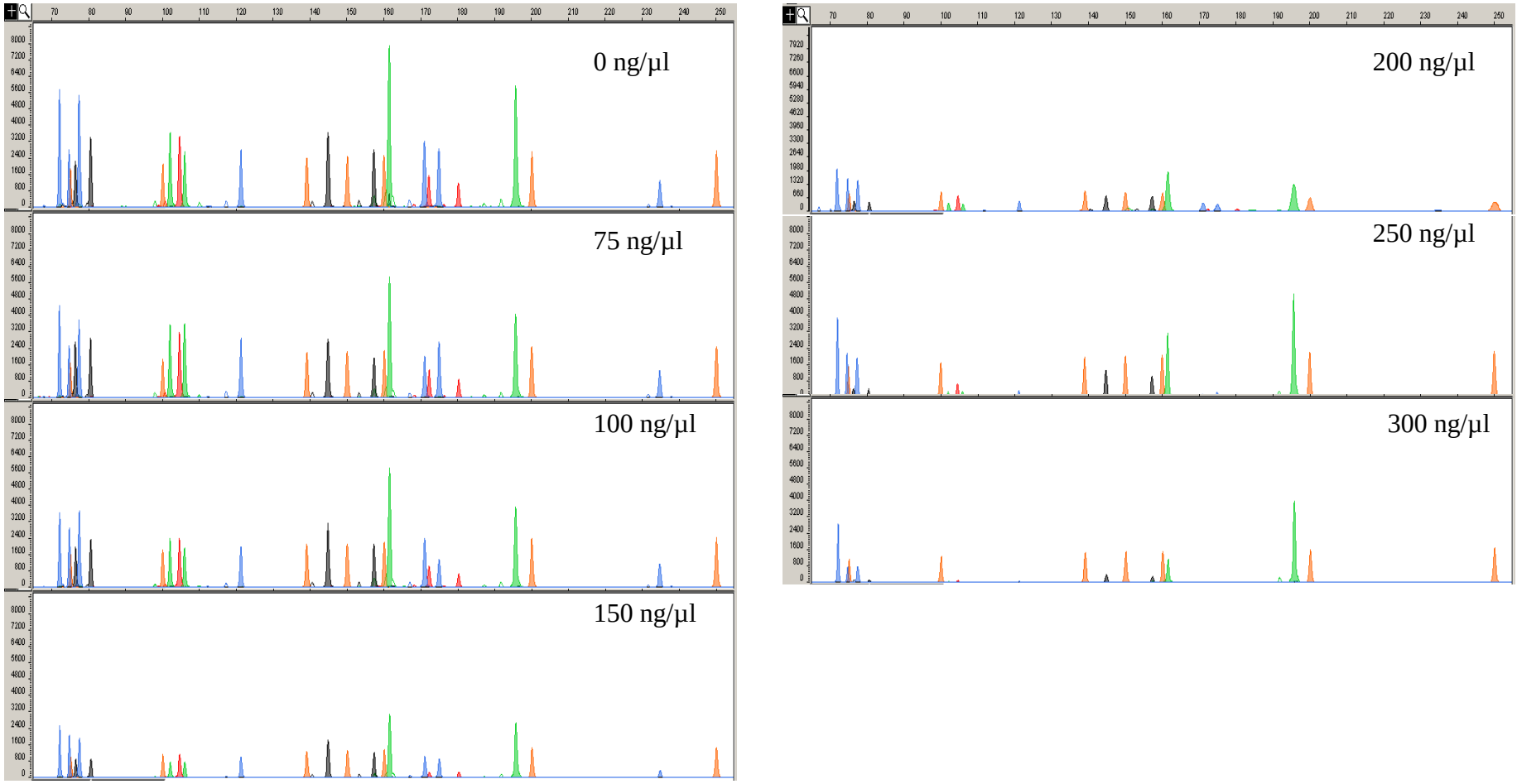


Figure 24. Representative results from stability study of tannic acid on Miniplex 1. Concentration of tannic acid were shown in each panel. With increasing concentration of tannic acid, locus dropout proceeded from the largest locus, DY392 before rest of the loci were affected. Allele dropouts were first observed at 200 ng/µl tannic acid. However, even at 250 ng/µl tannic acid, a complete DNA profile was still obtained.

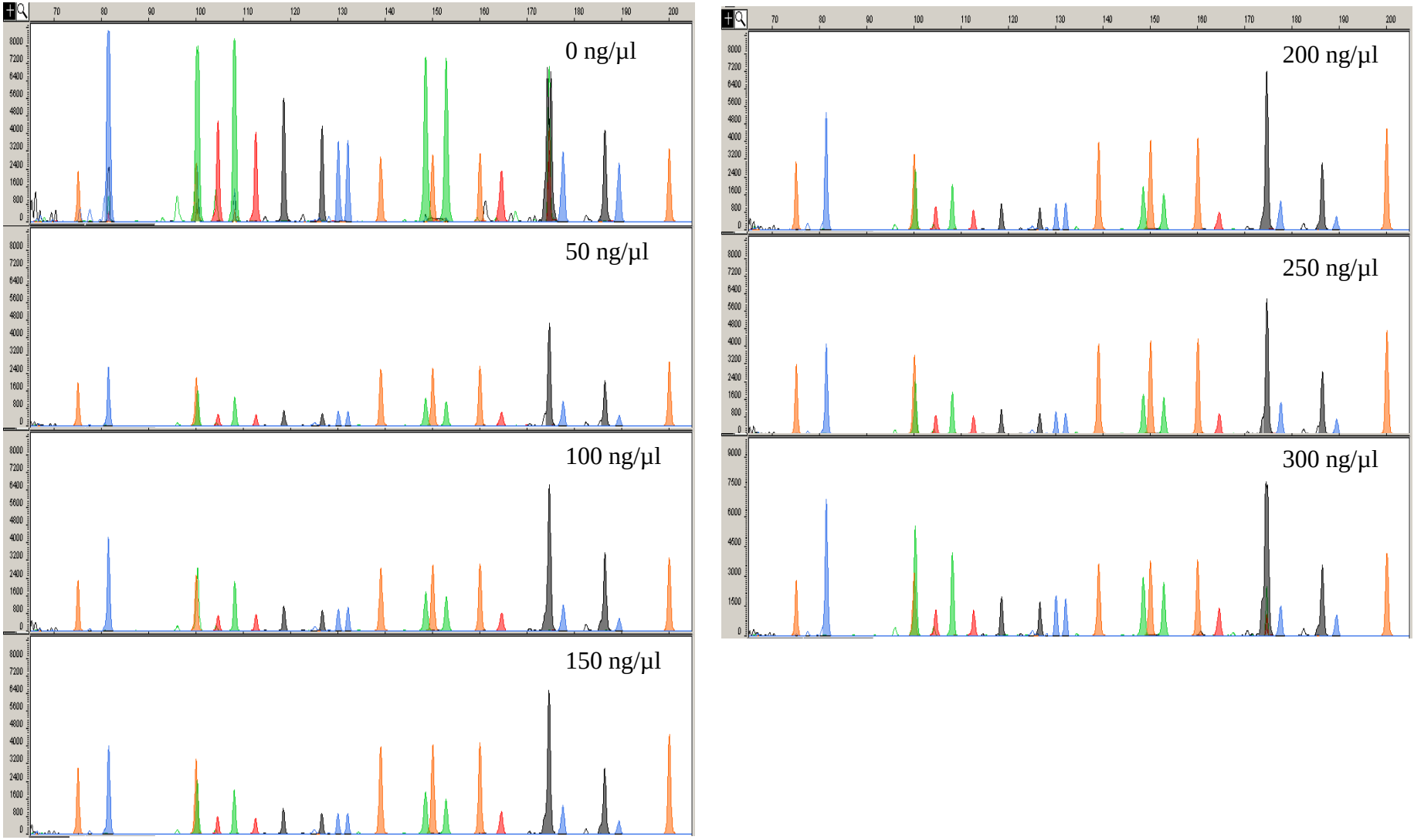


Figure 25. Representative results from stability study of tannic acid on Miniplex 2. Concentration of tannic acid were shown in each panel. With increasing concentration of tannic acid, efficiency of amplification remained consistent. Allele dropouts were observed in 250 ng/µl of tannic acid but at 300 ng/µl of tannic acid, full amplification was still achieved.

Table 8. Results from DNA samples challenged with increasing concentrations of tannic acid.

Kit	Tannic Acid (100 ng/μl)	Full amplification (%)	Average RFU	Average Peak Balance (%)
Miniplex 1 (n=3)	0	100	3847	84.6
	75	100	3461	83.1
	100	100	2984	79.5
	150	100	2357	85.1
	200	33	1311	88.8
	250	33	1402	80.1
	300	0	506	74.8
Miniplex 2 (n=3)	0	100	3532	79.0
	50	100	1390	76.1
	100	100	634	77.1
	150	100	483	76.1
	200	100	480	72.8
	250	50	350	64.6
	300	100	483	77.8

Percentage of full amplification (detection of all alleles in all loci), average RFU for all loci, and average peak balance for all loci were studied to study the effects of tannic acid inhibition on amplification.

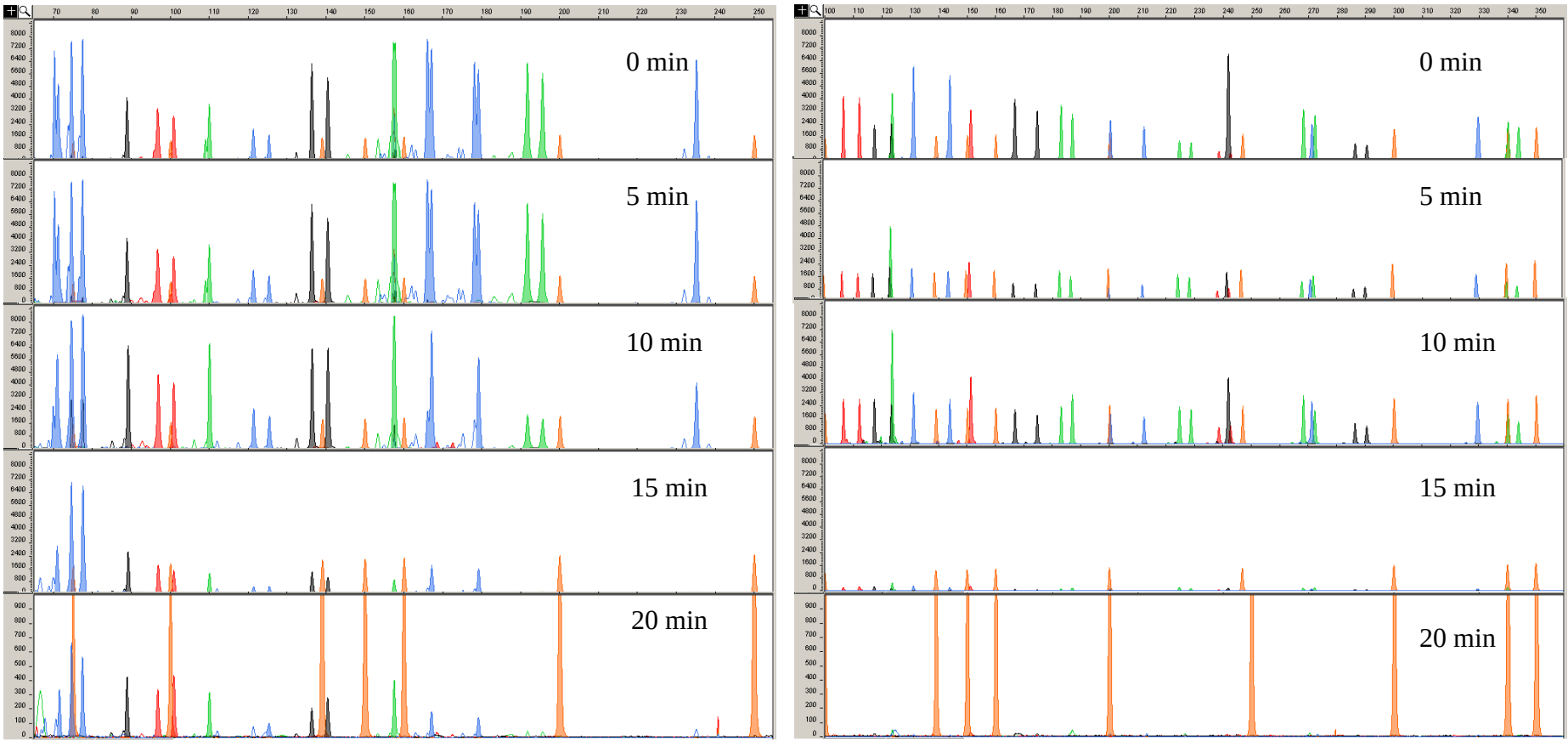


Figure 26. Representative results from degradation study between Miniplex 1 (panels on left) and Identifiler (panels on right). Incubation time with DNAse I were shown in each panel. At 20 minute incubation time for both Miniplex 1 and Identifiler®, the Y-scale is expanded for both set of results as amplification was compromised. Amplifications for both Miniplex 1 and Identifiler® were done in triplicates. R120 male genomic DNA was used in this study.

The stability studies using hematin, humic acid and tannic acid demonstrated an extremely high inhibitor tolerance of Miniplex 1 and 2, which would be ideal for forensics DNA typing analysis challenged with inhibitors. Compared to the Identifiler® multiplex kit, which our laboratory currently uses for forensic DNA typing which experiences amplification failure at 22 μM of hematin (Collins *et al.* 2004), the tolerance of Miniplex 1 and 2 to hematin is at least 15-fold higher than Identifiler®.

To demonstrate that the miniplex assay is more resistant to DNA template that is degraded, DNA template was subjected to Dnase I digestion for different time intervals. Only Miniplex 1 was compared to Identifiler® as the STR loci used in Miniplex 1 are the largest 8 STR loci found in Identifiler®. This is done to determine the recovery of the larger STR loci found in Identifiler® that are affected first when DNA template becomes smaller due to degradation.

As shown in Figure 26, the results for Miniplex 1 for time intervals 0, 5, and 10 minutes demonstrates over-amplification as 2ng of DNA was added to both Miniplex 1 and Identifiler®. 1ng of DNA as quantitated before DNA digestion was added for both multiplex systems at each time intervals to offset the loss of amplifiable DNA due to DNase I digestion. Surprisingly, FGA locus in Miniplex 1 was not detectable at all digestion time intervals, possibly inhibited by the high DNA template concentration, present either as digested and undigested forms. Full DNA profiles with the exception of FGA is obtained for Miniplex 1, while for Identifiler®, full DNA profiles were obtained at 0, 5 and 10 minutes time interval, full DNA profiles are obtained. At 15 minute interval, Identifiler® showed several losses of detectable alleles and at 20 minute interval, complete loss of detectable alleles was observed. The results demonstrated that Miniplex 1 was able to recover the loss of alleles that are experienced by Identifiler®, whenever the DNA template was highly

degraded which is consistent with studies done by other groups using MiniSTR primers to genotyped degraded DNA (Chung *et al.* 2004, Hummel *et al.* 1999, Alonso *et al.* 2001, Takahashi *et al.* 1997, Whitaker *et al.* 1995, Clayton *et al.* 1995).

5. Mixture

Table 9 and 10 describe the genotyping results for several mixture ratio of two male individuals at 10 loci for Miniplex 1 and 9 loci for Miniplex 2. The alleles contained in the table were based upon non-overlapping alleles determined from the non-mixed controls. Mixture study was conducted in which the total DNA input was maintained at 500 pg, and was designed to determine whether the two miniplex assays are able to detect low level mixtures, a scenerio which are found in forensic samples. Data from the experiments show that the minor component was present consistently at 10% of the total quantity of DNA template (50 pg minor contributor) for Miniplex 1. Genotypes from mixtures of 1:20 were detectable, but not without exceptions. For two replicates, the minor alleles (at CSF1PO, D7S820 and D13S317 locus) were either filtered away as it fell below the stutter threshold of the locus or the alleles were not detectable or under the detection threshold set at 50 RFU (at D2S1338, D21S11, D18S51, D16S539 and FGA locus). Therefore, when evaluating minor component alleles that fell in the stutter position of a major component, minor allele from 1:20 mixtures could be wrongly filtered as stutters, which indicates 1:20 as the lower limit of mixture detection for Miniplex 1.

For Miniplex 2, the experiments showed that the minor component were present consistently at 25% of the total quantity of DNA template (125 pg minor contributor).

Table 9 – Minor component genotype at non-overlapping alleles from replication amplification using Miniplex 1

Mixture Ratio	Miniplex 1 Locus				
	D2S133	D21S1	DYS39	CSF1P	D7S82
20:	18,19	29	14	10,13	11
10:	18,19	29	14	10,13	11
3:1	18,19	29	14	10,13	11
1:3	23,24	31	13	12	10
1:1	23,24	31	13	12	10
1:2	23,24	31	13	12	10
1:2	23,24	ND	13	*	10
1:2	ND	ND	13	*	*
Mixture Ratio	Miniplex 1 Locus				
	D13S31	TPOX	D18S5	D16S53	FGA
20:	8	8	17,18	12	21
10:	8	8	17,18	12	21
3:1	8	8	17,18	12	21
1:3	10	11	13,14	9,10	24
1:1	10	11	13,14	9,10	24
1:2	*	11	13,14	9,10	24
1:2	*	11	13,14	9.10	24
1:2	*	11	ND	ND	ND

*Allele present at stutter position of major component but below stutter filter threshold.
N.D:Alleles not detected (<50 RFU).
Detected genotype of minor component using a peak amplitude threshold of 50 RFU. Mixture ratio, which is displayed once as triplicate results are identical, established by reference sample (data not shown). Mixture ratio of 1:1 was also studied in triplicates as controls for complete amplification of all alleles from both reference samples mixed in equal proportions (data not shown). R120 and R60 male genomic DNA were used in this study.

Table 10 – Minor component genotype at non-overlapping alleles from replication amplification using Miniplex 2

Mixture Ratio	Miniplex 2 Locus								
	TH0	D19S43	D13S31	D3S135	D2S177	D5S81	vW	D8S117	DYS39
20:1	-	ND	ND	17	*	*	ND	11	21
20:1	-	ND	ND	17	*	*	17	11	21
20:1	-	14	ND	17	10	*	ND	11	21
10:1	-	14	8	17	10	10,12	17	11	21
3:1	-	14	8	17	10	10,12	17	11	21
1:3	10	13	10	-	12	11	16	10	24
1:10	ND	13	ND	-	12	11	*	*	ND
1:10	10	*	10	-	12	11	*	*	24
1:10	ND	*	ND	-	12	11	*	*	24
1:20	ND	ND	ND	-	ND	*	ND	*	ND
1:20	ND	ND	ND	-	ND	11	ND	*	ND
1:20	ND	*	ND	-	ND	*	ND	*	ND

*:Allele present at stutter position and below stutter filter threshold.
N.D:Alleles not detected (<50 RFU).
– Overlapped alleles between the 2 reference samples.
Detected genotype of minor component using a peak amplitude threshold of 50 RFU. Mixture ratio, which are displayed once as triplicate results are identical, established by reference sample (data not shown). Mixture ratio of 1:1 was also studied in triplicates as controls for complete amplification of all alleles from both reference samples mixed in equal proportions (data not shown). R120 and R60 male genomic DNA were used in this

Genotypes from mixtures of 1:10 were detectable, but not without exception. The minor alleles (at D19S433, vWA and D8S1179 locus) were either filtered away as it fell below the stutter threshold of the locus or the alleles were not detectable or under the detection threshold set at 50 RFU (at TH01, D13S317 and DYS390). Therefore, when evaluating minor component alleles that fell in the stutter position of a major component, minor allele from 1:10 mixtures could be mistakenly filtered as stutters, which indicates the lower limit of mixture detection for Miniplex 2. From the mixture studies, Miniplex 1 appeared to be a more robust assay in resolving DNA mixtures than Miniplex 2. The use of Y-STRs, DYS392 in Miniplex 1 and DYS390 in Miniplex 2 also demonstrate its usefulness in determining the number of male contributors in a mixed DNA profile.

6. Species Specificity

A range of animals were tested on both Miniplex 1 and 2. Amplification was observed mainly in primates, which included silver leaf langur, siamang, goeldies monkey, macaque, orang utan, long tail macaque, baboon, saki monkey, chimpanzee, gibbon, proboscis monkey, black howler monkey, javan langur, black spider monkey and douc langur. For minplex 1, only the slow loris did not show any amplification products, while 11 loci were amplified for the chimpanzee DNA. For Miniplex 2, silver leaf langur, goeldies monkey, slow loris, saki monkey, proboscis monkey, javan langur, black howler monkey and douc langur did not show any amplifications, while 6 loci were amplified for the chimpanzee DNA. Non-primate DNA included water buffalo, spotted hyena, Egyptian goose, German shepherd, red dhole, chicken, pig, goat, fish, pot-bellied pig and golden cat. For Miniplex 1, approximately, 72 to 79-bp sized 6-FAM fragments, which appeared as a single or three

distinct fragments and at reduced peak heights (<100 RFUs), at where the SRY and Amelogenin genotypes were expected. This peaks appeared in all mammals except the golden cat that are tested. These fragments can be attributed to SRY gene being conserved in all therians (Veyrunes *et al.* 2008), or a monomorphic product amplified from certain mammals by the amelogenin primers (Buel *et al.*1995). Three low level non-specific 6-FAM fragments, were found in german shepherd, red dhole and fish. The fragment size for german shepherd and red dhole, both canines were identical, at 124 bp while for the fish, at 120 bp. Both fragments were found within the D2S1338 alleles range and were marked as off-ladder alleles. Two low level VIC-fragments, sized at 177 and 199-bp were detected in spotted hyena and labeled as D7S820 allele 15 and D13S317 allele 12, respectively. No amplification products were detected for non-primates in Miniplex 2. The remainder of the tested species exhibited no amplification (chicken, golden cat, *P. aeruginosa*, *E. coli*, microbial pool and lambda DNA). Representative electropherograms for several species are illustrated in Figure 27.

Though amplification were observed in several non-primate species, the results were not surprising for mini-STRs as Opel and co-workers observed (Opel *et al.* 2007) observed amplification for D16S539 and D13S317 in non-human species. Amplification from non-human species, included primates could pose potential intepretation difficulties. The non-specific amplicons are documented and would serve as useful references when non-expected alleles appear in amplification. None of the non-human species were able to generate a full DNA profile using both miniplex assays, even for chimpanzee, and several loci are off-ladder alleles which would enable identification of non-human DNA being present.

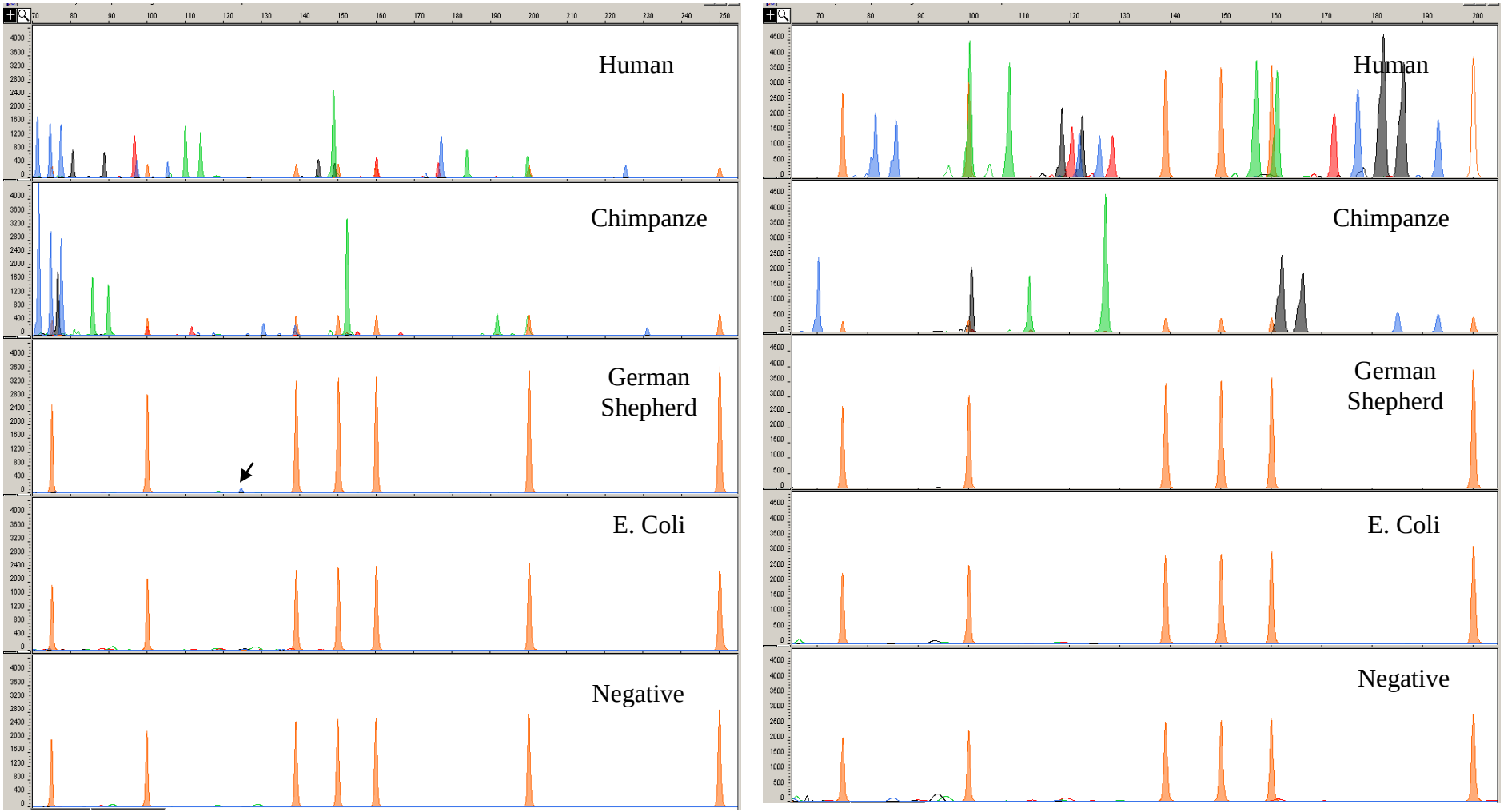


Figure 27. Representative results from a species specificity stud. Miniplex 1 (panels on left) and Miniplex 2 (panels on right). Arrow indicate the 124-bp size fragment artefact in caine DNA.

7. Standard specimens, concordance, reproducibility and population studies

All samples regardless of extraction technique used by the laboratory produced consistent and reproducible results with the Miniplex sets. NIST SRM 2391b, the PCR-based DNA profiling standard produced full concordance using the miniplex assays using Genomic 1 to 8 and G9947 DNA standards. In addition, 9948 and 007 male control DNA, which is standard positive controls for Promega and Applied Biosystems human identification multiplex kits were genotyped and full concordance was observed. This indicated the reporduciblity of both multiplexes. For FTA punch size of 1.2mm, the PCR cycle was adjusted from 30 cycles to 26 cycles in order to prevent over-amplification and causing the over-saturation of the charge coupled device (CCD) detector.

Miniplex 1 and 2 primer sets were earlier tested with a different buffer and enzyme combination i.e. Promega GoldS*TR and AmpliTaq Gold® DNA polymerase. PCR conditions were slightly different to the current miniplex assay, with annealing temperature set at 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute, in constrast to 59^{o}C for 2 minutes of annealing and 74^{o}C for extension for 1 minute in the present set-up. 238 Chinese, 241 Malays and 243 Indians for Miniplex 1, while for Miniplex 2, 249 Chinese, 244 Malays and 248 Indians were earlier genotyped. Since the same primer sets were used, a subset of the earlier samples was taken to be re-genotyped with the current miniplex assay for concordance. For Miniplex 1, 83 Chinese, 78 Malays, 82 Indians were re-genotyped while for Miniplex 2, 81 Chinese, 77 Malays and 90 Indians were re-genotyped using the present miniplex assays. The results are summarised in Table 11.

No discordant results were observed among the Chinese either for the gender or STR loci in both miniplex assays. For Malays, there were 25 discordant D7S820 allele calls out of 78

Table 11. Summary of 53 discordant STR profiling results observed in this study between Identifiler kit and our Miniplex assays for 34 Malays and 19 Indians. No discordant results for Chinese. * denotes miniplex assays using Promega GoldST*R buffer and AmpliTaq Gold DNA polymerase with annealing temperature at 55^{o}C. 5 Malay samples have dual discordant results.

	Locus	Origin	Identifiler	*Miniplex 1,2	Miniplex 1,2	Likely Cause
1.	D7S820	Malay	**8**.12	8,12	12,12	Allele 8 primer binding mutation
2.	D7S820	Malay	**9**,11	9,11	11,11	Allele 9 primer binding mutation
3.	D7S820	Malay	**8**,11	8,11	11,11	Allele 8 primer binding mutation
	D16S539		**8**,9	9,9	9,9	Allele 8 primer binding mutation
4.	D7S820	Malay	8,**11**	8,**11**	8,8	Allele 11 primer binding mutation
5.	D7S820	Malay	**8**,10	**8**,10	10,10	Allele 8 primer binding mutation
6.	D7S820	Malay	11,**12**	11**,12**	11,11	Allele 12 primer binding mutation
7.	D7S820	Malay	**8**,10	**8**,10	10,10	Allele 8 primer binding mutation
8.	D7S820	Malay	**8**,11	**8**,11	11,11	Allele 8 primer binding mutation
9.	D7S820	Malay	**8**,11	**8**,11	11,11	Allele 8 primer binding mutation
10.	D7S820	Malay	**8**,11	**8**,11	11,11	Allele 8 primer binding mutation
	Amelogenin		X,X	X, X	X, X	SRY, DYS390 and DYS392 alleles detected. Amel Y primer binding mutation or Yp11.2 deletion.
11.	D7S820	Malay	**8**,11	**8**,11	11,11	Allele 8 primer binding mutation
12.	D7S820	Malay	**8**,10	**8**,10	10,10	Allele 8 primer binding mutation
13.	D7S820	Malay	**8**,12	**8**,12	12,12	Allele 8 primer binding mutation
	Amelogenin		X,X	X,X	X,X	SRY, DYS390 and DYS392 alleles detected. Amel Y primer binding mutation or Yp11.2 deletion.
14.	D7S820	Malay	**9**,11	**9**,11	11,11	Allele 9 primer binding mutation
15.	D7S820	Malay	**8**,10	**8**,10	10,10	Allele 8 primer binding mutation
16.	D7S820	Malay	**8**,12	**8**,12	12,12	Allele 8 primer binding mutation
17.	D7S820	Malay	**8**,12	**8**,12	12,12	Allele 8 primer binding mutation
18.	D7S820	Malay	**10**,11	**10**,11	11,11	Allele 10 primer binding mutation
19.	D16S539	Malay	**8**,9	9,9	9,9	Allele 8 primer binding mutation
20.	D7S820	Malay	11,**12**	11,**12**	11,11	Allele 12 primer binding mutation

Table 11: Continue.

	Locus	Origin	Identifiler	*Miniplex 1,2	Miniplex 1,2	Likely Cause
21.	D7S820	Malay	**8**.11	8,11	11,11	Allele 8 primer binding mutation
	D16S539		**8**,13	13,13	13,13	Allele 8 primer binding mutation
22.	D7S820	Malay	10,**11**	10,**11**	10,10	Allele 11 primer binding mutation
23.	D7S820	Malay	8,11	8,11	**8**,11	Allele 8 primer binding mutation
24.	D7S820	Malay	**8,**12	**8**,12	12,12	Allele 8 primer binding mutation
25.	D7S820	Malay	**9**,11	**9**,11	11,11	Allele 9 primer binding mutation
26.	D7S820	Malay	**8,**11	**8**,11	11,11	Allele 8 primer binding mutation
	D16S539		**8**,10	10,10	10,10	Allele 8 primer binding mutation
27.	D16S539	Malay	**8**,11	11,11	-	Allele 8 primer binding mutation
28.	Amelogenin	Malay	X,X	X,X	-	SRY, DYS390 and DYS392 alleles detected. Amel Y primer binding mutation or Yp11.2 deletion.
29.	D16S539	Malay	**8**,12	12,12	-	Allele 8 primer binding mutation
30.	D16S539	Malay	**8**,9	9,9	-	Allele 8 primer binding mutation
31.	D16S539	Malay	**8**,13	13,13	-	Allele 8 primer binding mutation
32.	D16S539	Indian	**8**,11	11,11	11,11	Allele 8 primer binding mutation
33.	Amelogenin	Indian	X,X	X,X	X,X	SRY, DYS390 and DYS392 alleles detected. Amel Y primer binding mutation or Yp11.2 deletion.
34.	D16S539	Indian	**8**,12	12,12	12,12	Allele 8 primer binding mutation
35.	D16S539	Indian	**8,**12	12,12	12,12	Allele 8 primer binding mutation
36.	D16S539	Indian	**8,**12	12,12	12,12	Allele 8 primer binding mutation
37.	D16S539	Indian	**8**,10	10,10	10,10	Allele 8 primer binding mutation
38.	D16S539	Indian	**8**,11	11,11	11,11	Allele 8 primer binding mutation
39.	D16S539	Indian	**8**,10	10,10	10,10	Allele 8 primer binding mutation
40.	D16S539	Indian	**8**,11	11,11	11,11	Allele 8 primer binding mutation
41.	D16S539	Indian	**8**,9	9,9	-	Allele 8 primer binding mutation

Table 11: Continue.

	Locus	Origin	Identifiler	*Miniplex 1,2	Miniplex 1,2	Likely Cause
42.	Amelogenin	Indian	X,X	X,X	-	SRY, DYS390 and DYS392 alleles detected. Amel Y primer binding mutation or Yp11.2 deletion.
43.	D16S539	Indian	**8**,13	13,13	-	Allele 8 primer binding mutation
44..	Amelogenin	Indian	X,X	X,X	-	SRY, DYS390 and DYS392 alleles detected. Amel Y primer binding mutation or Yp11.2 deletion.
45.	D16S539	Indian	**8**,11	11,11	-	Allele 8 primer binding mutation
46.	D16S539	Indian	**8**,11	11,11	-	Allele 8 primer binding mutation
47.	D16S539	Indian	**8**,11	11,11	-	Allele 8 primer binding mutation
48.	TPOX	Indian	8,**11**	8,8	-	Allele 11 primer binding mutation
49..	Amelogenin	Indian	X,X	X,X	-	SRY, DYS390 and DYS392 alleles detected. Amel Y primer binding mutation or Yp11.2 deletion.
50.	D16S539	Indian	**8**,12	12,12	-	Allele 8 primer binding mutation
51	D16S539	Indian	**8**,11	11,11	-	Allele 8 primer binding mutation
52.	D16S539	Indian	**8**,12	12,12	-	Allele 8 primer binding mutation
53.	D16S539	Indian	**8**,14	14,14	-	Allele 8 primer binding mutation
54.	DYS390	Malay	-	Null	Null	Primer binding mutation
55.	TH01	Malay	**7**,9.3	9.3,9.3	-	Allele 7 primer binding mutation

Malay samples between the present Miniplex 1 and Identifiler, while the previous Miniplex 1 did not show any discordant D7S820 allele calls. This difference was due to annealing temperatures between the two miniplex 1 assays as the present primer set used 59^{o}C as the annealing temperature, and not the lower 55^{o}C annealing temperature used in the earlier assay. This observation is consistent with reports that have demonstrated recovery of null or imbalance alleles in multiplex when annealing temperature were lowered (Forrest *et al.* 2004, Leibelt *et al.* 2003). Out of the 25 discordant D7S820, 17 were for allele 8, indicating that the primer binding mutation is closely linked with the allele. The primer mutation at D7S820 was observed only among Malays. This same phenomenon has been reported for D8S1179 locus in Chamorros and Filipinos from Guam were found to have primer binding mutation using Profiler Plus multiplex assay from Applied Biosystems with 5/68 Chamorros and 8/72 Filippino samples being affected resulting in false homozygotes (Budowle *et al.* 2001). For D7S820, 25/77, or 32% of the samples genotyped has false homozygosity. This percentage is of concern as discordance can result in mis-identification when different multiplex assays are utilized. To overcome the non-binding primer, a second amplification can be performed using a lower annealing temperature or degenerate primers that have offset the primer binding mutation can be added to Miniplex 1 primer set. This will require the D7S820 loci to be sequenced in order to identify the base mutation responsible for the null allele. For D8S1179 in Chamorros and Filipinos populations, an extra primer to account for the variant primer binding site was added to the Profiler Plus primer set without any deleterious impact on the genotyping (Leibeit *et al.* 2003). Alternatively a lower annealing temperature of 55^{o}C or even lower can be used to recover the null allele.

Discordant genotype at D16S539 locus was also observed. Using Miniplex 1, 8 out of 241 (3%) Malays and 17 out of 243 (7%) Indians regardless of the current or previously utilized minplex assays were found to have a null allele 8 when compared with Identifiler genotypes. Similarly to

D7S820, the same approach of adding additional primers to include the variant primer binding locations can be used to resolve the false homzygosity at D16S539 or amplification. For D16S539, it appeared that the variant primer binding mutation is specific for allele 8 unlike D7S820 which includes other alleles and the mutation is most likely at the 3' terminal end of the primer sequence since as using both annealing temperatures of 55^{o}C and 59^{o}C, allele 8 was not genotyped. This possiblity would require confirmation by sequencing. One instance of discordant null allele in TH01, TPOX and DYS390 in the STR loci were observed in the concordance studies, which would be due to primer mutations at the binding site on the DNA template.

For amelogenin, discordant gender was found in 3 out of 210 (1.4%) Malay males and 4 out of 219 (1.8%) Indian males, when DNA profiles were compared between Identifiler and the miniplex assays. All the samples in Identifiler were mistyped as females while both miniplex assays correctly identified as males. The miniplex assays, which use a panel of Y-specific markers, namely SRY, amelogenin (different primer set from Identifiler) and Y-STRs (DYS390 and DYS392) enabled reliable gender genotyping. Identifiler and other commercially available STR typing kits based gender identification singularly on the amelogenin test.

Reliable gender identification is important in forensic investigations as wrong gender information could mislead investigators as well as mis-identification of mass disaster victims. Amelogenin nulls have been reported among Malays and especially for Indians. Chang and co-workers (2003) have reported 1 out of 113 Malays and 4 out of 112 Indians in Malaysia having amelogenin Y nulls. Cadenas and co-workers (2007) have reported 5 out of 77 Kathmandu males having dropout of the amelogenin Y allele while Thangaraj *et al.* 2002 reported 1.85% of Indians have amelogenin deletions. Our laboratory had also observed amelogenin null among Bangladeshi, Chinese and Malay individuals (Lim *et al.* 2005). A more recent report by Chang and co-workers (2007) found

3.2% of Indians and 0.6% of Malays are amelogenin Y negative males. Similar amelogenin nulls were reported in Australia (Roffey *et al.* 2000), Austria (Steinlechner *et al.* 2002), Israeli (Michael and Brauner 2004) and Japanese (Takayama *et al.* 2009) populations. Most of the reports indicated the null amelogenin Y genotype due to Yp11.2 deletions involving the amelogenin gene, and to a much lesser extent, primer binding mutation.

Additional Y-specific markers have been used to confirm gender in forensic investigations. This inclued SRY (Kastelic *et al.* 2009), 2 different amelogenin primer sets in combination with SRY (Esteve Codina *et al* 2009), Y-STRs (Takayama *et al.* 2009, von Wurmb-Schwark *et al.* 2009) or a combination of the three approaches (Lim *et al.* 2005). In this study, the employment of SRY, alternative amelogenin primer sets and 2 Y-STR specific loci DYS390 and DYS392 produced reliable male gender identification. Full concordance between Identifiler and both miniplex assays was observed in 99.8% (24,878 out of 24,928) allele calls compared. SRY, DYS390, DYS392 and D2S1776 were not compared since these markers are not found in Identifiler.

For population studies, Identifiler STR markers were not examined since the population studies for these markers have been completed (Budowle, 2004, Syn *et al.*, 2005, Ang *et al.* 2005 and Lim *et al.* 2005). For DYS390 and DYS392, the population data of Singapore was also published (Budowle *et al.* 2009, Tang *et al.* 2006). D2S1776 was examined using PowerStats and the summary of the population studies is summarized in Table 12. Based on the heterozygosity values of 78.5%, 63.8% and 69.5% among the Chinese, Malay and Indian, D2S1776 is comparable to the heterozygosity values for STR markers in Identifiler®. (Ang *et al.* 2005, Budowle *et al.* 2009, Lim *et al.* 2005, Syn *et al.* 2005)

Table 12. Summary of D2S1776 population statistics using PowerStats.

Locus:	**D2S1776**		
	Chinese	**Malay**	**Indian**
Forensic			
Matching Probability	0.068	0.148	0.127
Expressed as 1 in ...	14.7	6.8	7.9
Power of Discrimination	0.932	0.852	0.873
PIC	0.77	0.64	0.66
Paternity			
Power of Exclusion	0.572	0.339	0.420
Typical Paternity Index	2.33	1.38	1.64
Allele Frequencies			
Homozygotes	21.5%	36.2%	30.5%
Heterozygotes	78.5%	63.8%	69.5%
Total Alleles	494	492	498

Allele	**Frequency**		
7	0.2%	0.2%	
8	28.7%	0.8%	2.4%
9	13.6%	3.9%	3.2%
10	15.4%	5.5%	7.4%
10.1			0.2%
11	24.5%	28.3%	31.1%
12	12.3%	46.3%	41.8%
13	4.3%	12.6%	11.6%
14	1.0%	2.2%	1.6%
15		0.2%	0.6%

8. Simulated Forensic Samples

Our laboratory had previously prepared mock forensic samples for validation of a DNA extraction protocol using Promega Maxwell® 16 DNA extraction system (Lim *et al.* 2009).

In addition, unused samples from our external proficiency testing were also used. Organic extracted DNA from these samples was amplified to evaluate Miniplex 1 and 2 performance in comparison to the genotypes obtained using Identifiler®. The summary of the results are found in Table 13. A total of 76 samples were examined, which comprised of liquid blood, cigarettes, bloodstains on cloth, leather, shirt, soil, blue denim jeans and swabs stained with semen.

From table 13, 44 out of the 74 samples genotyped, full DNA profiles were obtained using Identifiler®. Similarly, Miniplex 1 and 2 were able to obtain complete DNA profile. The DNA profile from the remaining 30 samples were found to have either partial or no DNA profile was obtained using Identifiler. Of these samples, 6 out of 30 samples managed to produce full DNA profiles using both Miniplex 1 and 2. With the exceptions of 5 samples obtained from soil, which did not generate any DNA profiles, the remaining samples recovered more alleles using Miniplex 1 and 2 than Identifiler®. The results demonstrated that Miniplex 1 and 2 were able to obtain significantly more alleles than Identifiler®, when samples are present in low quantity, degraded and inhibited. The non-amplification from soil is either due to high inhibitor concentration or DNA is fully degraded.

However, abnormalities that were unexpected were encountered in 4 samples that did not yield any DNA profile using Identifiler®. Though successful genotyping were obtained using Miniplex 1 and 2, there were non-specific amplification products using Miniplex 1.These 4 samples shared one thing in common in that using Identifiler®, these samples showed non-amplification, most likely due to the presence of high amounts of inhibitors in the DNA extracts. For samples derived

Table 13. Summary of simulated casework genotyped using Miniplex 1 and Miniplex 2. For Miniplex 1, if sample is of female origin, only 10 loci can be genotyped since SRY and DYS392 are male specific loci. For Miniplex 2, if sample is of female origin, 8 loci can be genotyped since DYS390 are male specific loci. Samples that were not tested were due to insufficient DNA extract. Samples with allele dropouts using Identifiler are **bold**.

Sample Marking	**Type of Sample**	**No of loci detected / No of loci in multiplex assay**			**Remarks**
		Identifiler	Miniplex 1	Miniplex 2	
Item_ 1_ 031007	Blood Stain on Cloth	16/16	10/10	8/8	
Item_ 2_ 031007	Blood Stain on Cloth	16/16	12/12	9/9	
Item_ 3_ 031007	Blood Stain on Cloth	16/16	10/10	8/8	
Item_ 4_ 031007_Counch Stain	Blood Stain on Cloth	16/16	12/12	9/9	
BIS18_031007	Blood Stain on Cloth	16/16	10/10	8/8	
Item_1_090707	Blood Stain on Cloth	16/16	10/10	8/8	
Item_2_090707	Blood Stain on Cloth	16/16	12/12	9/9	
Item_3_090707_MF	Blood and Semen Stain on Cloth	16/16	12/12	9/9	
Item_3_090707_FF	Blood and Semen Stain on Cloth	16/16	12/12	9/9	
Item_4_090707	Blood Stain on Cloth	16/16	12/12	9/9	
Item_ 1_ 080107	Blood Stain on Cloth	16/16	10/10	8/8	
Item_ 2_ 080107	Blood Stain on Cloth	16/16	12/12	9/9	
Item_ 3_ 080107	Blood Stain on Cloth	16/16	12/12	9/9	
Item_4_080107	Blood Stain on Cloth	16/16	12/12	9/9	
BIS17_080107	Blood Stain on Cloth	16/16	10/10	8/8	
Item_1_290307	Blood Stain on Cloth	16/16	10/10	8/8	
Item_2_290307	Blood Stain on Cloth	16/16	12/12	9/9	
Item_3_290307_MF	Blood and Semen Stain on Cloth	16/16	12/12	9/9	
Item_3_290307_FF	Blood and Semen Stain on Cloth	16/16	12/12	9/9	
Item_4_290307	Blood Stain on Cloth	16/16	10/10	8/8	
A_Shirt	Blood Stain on Shirt	16/16	12/12	9/9	
B_Shirt	Blood Stain on Shirt	16/16	10/10	8/8	
C_Shirt	Blood Stain on Shirt	16/16	10/10	8/8	
D_Shirt	Blood Stain on Shirt	16/16	10/10	8/8	
E_Shirt	Blood Stain on Shirt	16/16	12/12	9/9	

Table 13. Continued.

Sample Marking	Type of Sample	No of loci detected / No of expected loci in multiplex assay			Remarks
		Identifiler	Miniplex 1	Miniplex 2	
A_Leather	Blood Stain on Leather Belt	16/16	12/12	9/9	
B_Leather	Blood Stain on Leather Belt	16/16	10/10	8/8	
C_Leather	Blood Stain on Leather Belt	16/16	10/10	8/8	
D_Leather	Blood Stain on Leather Belt	16/16	10/10	8/8	
E_Leather	Blood Stain on Leather Belt	16/16	12/12	9/9	
R20	Liquid Blood	16/16	12/12	9/9	
R21	Liquid Blood	16/16	10/10	8/8	
R29	Liquid Blood	16/16	10/10	8/8	
R30	Liquid Blood	16/16	10/10	8/8	
R31	Liquid Blood	16/16	10/10	8/8	
R34	Liquid Blood	16/16	10/10	8/8	
R36	Liquid Blood	16/16	10/10	8/8	
R39	Blood stain on Cloth	16/16	10/10	8/8	
R40	Blood stain on Cloth	16/16	10/10	8/8	
C1_20-1	Cigarette Butt	16/16	12/12	9/9	
C2_20-1	Cigarette Butt	16/16	12/12	Not Tested	
C7_24-1	Cigarette Butt	16/16	10/10	8/8	
C8_24-1	Cigarette Butt	16/16	10/10	8/8	
A50x20_1	Blood diluted 50x and stained	11/16	10/12	9/9	
A100x20_1	Blood diluted 100x and stained	3/16	11/12	9/9	
A500x_20_1	Blood diluted 500x and stained	0/16	9/12	9/9	
B500x20_1	Blood diluted 500x and stained	5/16	9/10	8/8	

Table 13. Continued.

Sample Marking	Type of Sample	No of loci detected / No of expected loci in multiplex assay			Remarks
		Identifiler	Miniplex 1	Miniplex 2	
A_Shirt_20_1	Blood stain on shirt	13/16	12/12	9/9	
A_Denim_20_1	Blood stain on blue jeans	0/16	12/12	9/9	~209 bp sized FAM fragment detected in Miniplex 1 ~168 bp VIC sized fragment detected in Miniplex 1
B_Denim_20_1	Blood stain on blue jeans	0/16	8/10	4/8	~112 bp and 132 bp sized FAM fragments detected in Miniplex 1
C_Denim_20_1	Blood stain on blue jeans	0/16	10/10	8/8	
D_Denim_20_1	Blood stain on blue jeans	0/16	10/10	8/8	
E_Denim_20_1	Blood stain on blue jeans	0/16	9/12	2/9	~123 bp and 209 sized FAM fragments detected in Miniplex 1 ~168 bp VIC sized fragment detected in Miniplex 1
A_Soil_20_1	Blood stain on soil	0/16	0/12	0/9	
B_Soil_20_1	Blood stain on soil	0/16	0/10	0/8	
C_Soil_20_1	Blood stain on soil	0/16	0/10	0/8	
D_Soil_20_1	Blood stain on soil	0/16	0/10	0/8	
E_Soil_20_1	Blood stain on soil	0/16	0/10	0/8	
C3_20_1	Cigarette Butt	11/16	12/12	Not Tested	
C4_20_1	Cigarette Butt	12/16	12/12	9/9	
C5_20_1	Cigarette Butt	15/16	12/12	9/9	
C6_24_2	Cigarette Butt	12/16	12/12	9/9	
C9_24_2	Cigarette Butt	0/16	12/12	Not Tested	~168 bp sized VIC fragment detected in Miniplex 1 ~170 bp sized NED fragment detected in Miniplex 1

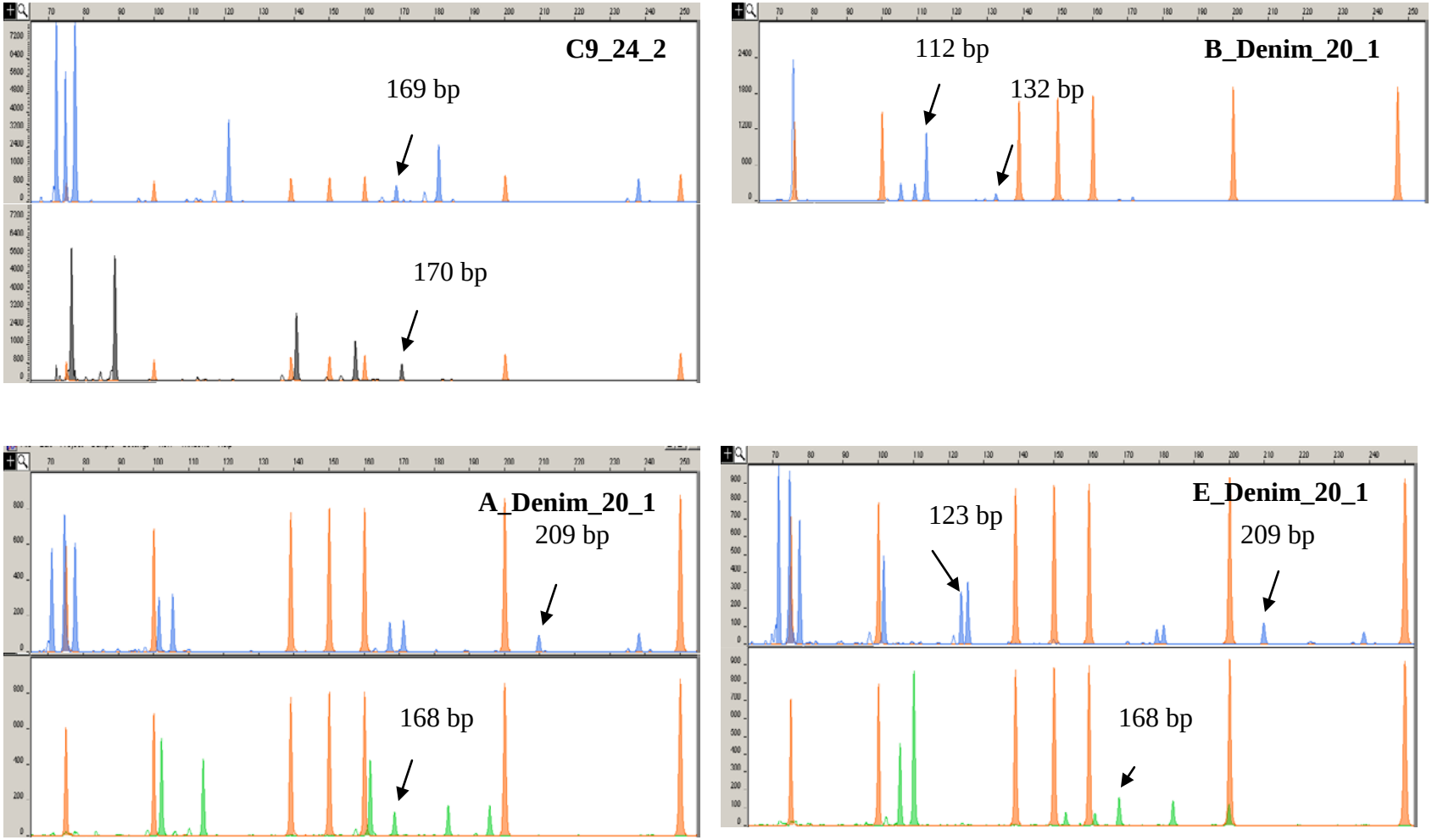


Figure 28. Non-specific amplification fragments of simulated casework samples that are highly inhibited. The non-specific amplification fragments are pointed by an arrow and the fragment size given and sample markings are indicated in panel.

from denim jeans, indigo blue dye is known to be an inhibitor of PCR (Shutler *et al.* 1999). For cigarette, it is likely the presence of humic acid or tanic acid from tobacco material that would have cause PCR inhibition (Tsai *et al.* 1992 and Watson and Blackwell *et al.* 2000), however the nature of inhibitors present were not ascertained though the presence of inhibitors were indicated by the data obtained from Quantifiler®. The artifacts of the 4 samples are highlighted in the electrophoregrams in Figure 28.

The use of Miniplex 1 managed to obtain a full or partial DNA profile but the occurrence of unexpected fragments posed issues towards interpretation of the DNA profiles. Two of the fragments were reproducible, namely the 209 and 168 – bp sized fragments in 6'FAM and VIC panels, which would allow easy identification with its appearance in inhibited DNA samples. The 123 bp sized 6'FAM fragment is the most disconcerting as it was assigned allele 28.2 in the D21S11 locus. The remaining fragments are found at 112, 169 and 132 bp position in 6'FAM panel, while the 170 bp sized fragment were found in the NED panel. All these fragments are off-ladders and were found within the loci D2S1338, D21S11, D7S820 and D18S51. Their appearance could be due to several reasons. One of which could be the presence of strong inhibitors which could have a detrimental effect on the specificity of the OmniTaq Polymerase though this non-specific amplification was not observed in Miniplex 2. OmniTaq Polymerase is a mutant Taq polymerase at codon 708, which conferred enhanced resistance to various inhibitors (Kermekchiev *et al.* 2009). This mutation might under inhibitory conditions compromise its specificity though tests would need to be performed to determine this. In addition, Miniplex 1 had more loci being amplified than Miniplex 2 and there were more primer to primer interactions and this could be a contributory factor why non-specific amplification appeared under severe inhibitory conditions with unknown inhibitors. As studies

have been carried out on common inhibitors without any artifacts being formed, the appearance of these fragments might also be due to other factors, which needed to be studied and solutions can then be proposed for its remedy. The STR loci that were affected, namely D2S1338, D21S11, D7S820 and D18S51 should be interpreted with caution and verification procedures such as re-amplification or alternative primer set amplification can be performed to establish the true alleles whenever it is encountered. Mixture intepretation would pose as a problem as accurate intepretation would be difficult with non-specific fragments appearing. Miniplex 2, however did not encounter such non-specific fragments which indicates its reliabilty and ease of intepretation. Though the non-specific amplifications in Miniplex 1 would make intepretation difficult, the ability to amplify highly inhibited samples would still be useful for human identification, and with careful interpretation, also be useful genotype information could still be obtained.

3. Initial Development of Miniplex C

Multiplex genotyping produced DNA profile of similarly sized amplicons being resolved by having large size differences (~100 bp to 400 bp) and being tagged by different fluorescent dyes. The development of a 5 dyes system from a 4 dye system have allowed more similarly sized amplicons to be genotyped. The 5-dye system can accommodate 4 identically sized fragments resolved by the 4 dye colours, the remaining color acting as an internal size standard. However, when degraded DNA is encountered, amplicons size would need to be reduced to accommodate the smaller available DNA template for amplication. This has resulted in miniSTR primers being proposed and developed into several miniplexes in order to cover all CODIS markers (Bulter *et al.* 2003). The use of multiple miniplexes is not always favourable when limited amount of DNA

is available and with degraded DNA, as identical small-sized amplicons of less than 200 bp were produced. This makes the fragment size identical in lengths and several miniplexes are needed to gather enough STR genotypes for useful identification since they occupy the same size window and the only course of resolution is by dye-color. Hence, if more miniSTR primers produced amplicons can be resolved in single genotyping attempt; this would circumvent the limitation imposed by the amount of DNA available.

In order to increase the resolving ability of the multiplexes, a novel solution was proposed. The forward primer set for Miniplex 1 was modified to contain an internal Uracil which replaced a Thymine nucleotide in the primer sequence. The forward primer set for Miniplex 2 was modified to contain an internal Inosine which replaced a Thymine nucleotide in the primer sequence. The use of a Uracil or Inosine specific cleavage enzyme would remove amplicons produced by Miniplex 1 or Miniplex 2, respectively. This approach would double the number of STR loci with identical sized amplicons being genotyped in a single amplification. Suitable cleavage enzymes were tested and eventually USER^{TM} enzyme, a Uracil specific cleavage enzyme and *Thermotoga maritima* thermastable endonuclease V, an Inosine specific cleavage enzyme were selected. *E. coli* endonuclease V were also tested but was dropped due to its non-specificity in cleaving inosine-containing amplicons as uracil-containing amplicons were partially cleaved, resulting in reduced signal strength (data not shown). The flow diagram of this new approach is shown in Figure 29.

A total of 9 samples were tested in using this approach. Samples include 9947, 9948, 007 and 6 DNA extracts from neat blood samples were utilised to determine the viablity of this approach. All 9 samples showed that co-amplication using both Miniplex 1 and 2 is viable and cleaving an aliquot of 5 µl of PCR product with either USER^{TM} enzyme or Endonuclease V enzyme,

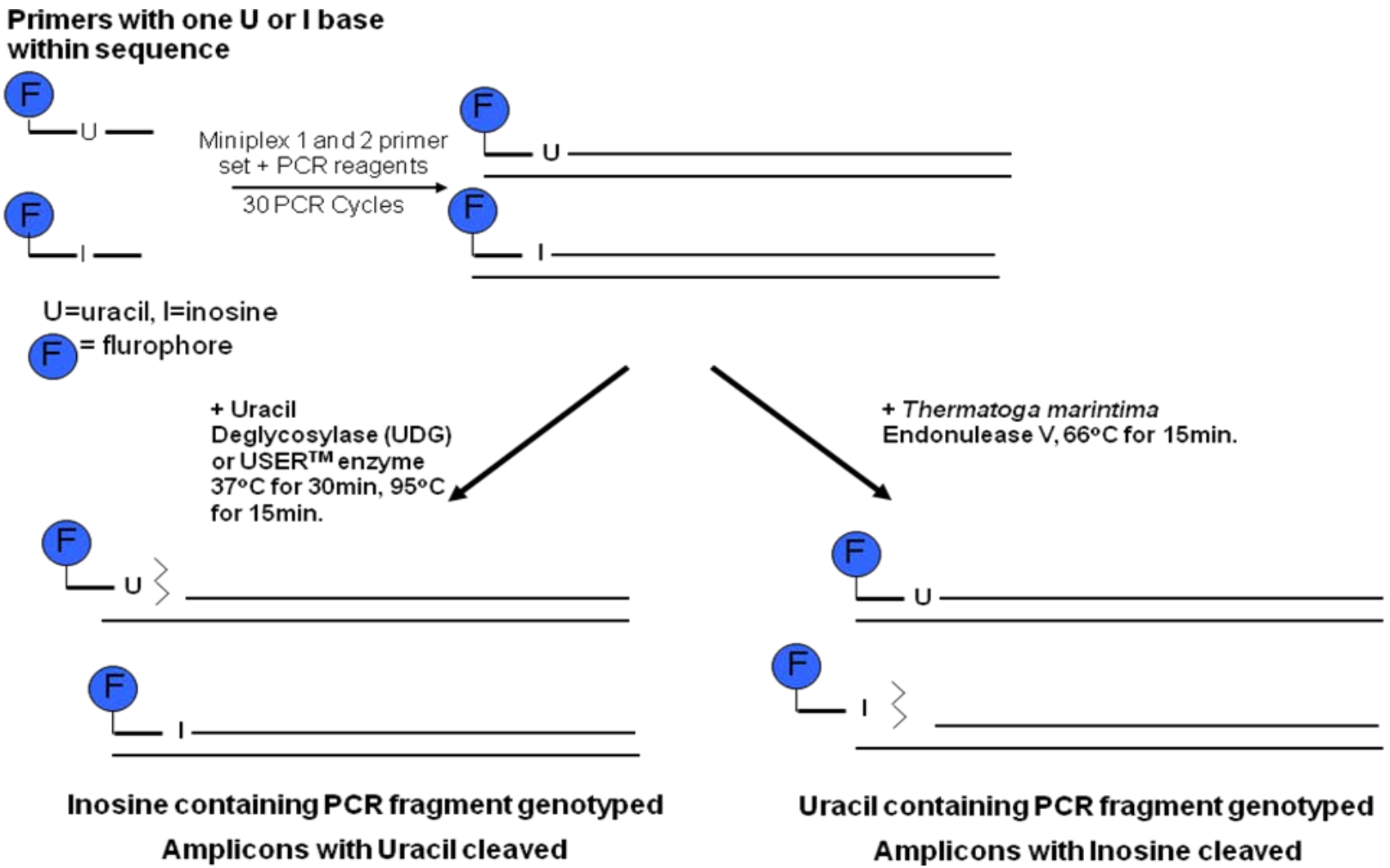


Figure 29. Flow diagram of the genotyping strategy used by Miniplex C. Miniplex C uses both Miniplex 1 and 2 primer sets in 1 PCR amplification reaction. To genotype each set, either USER™ enzyme or Endonuclease V is added. When USER™ enzyme is added, only inosine containing amplicons would be genotyped and When Endonuclease V is added, only uracil containing amplicons would be genotyped.

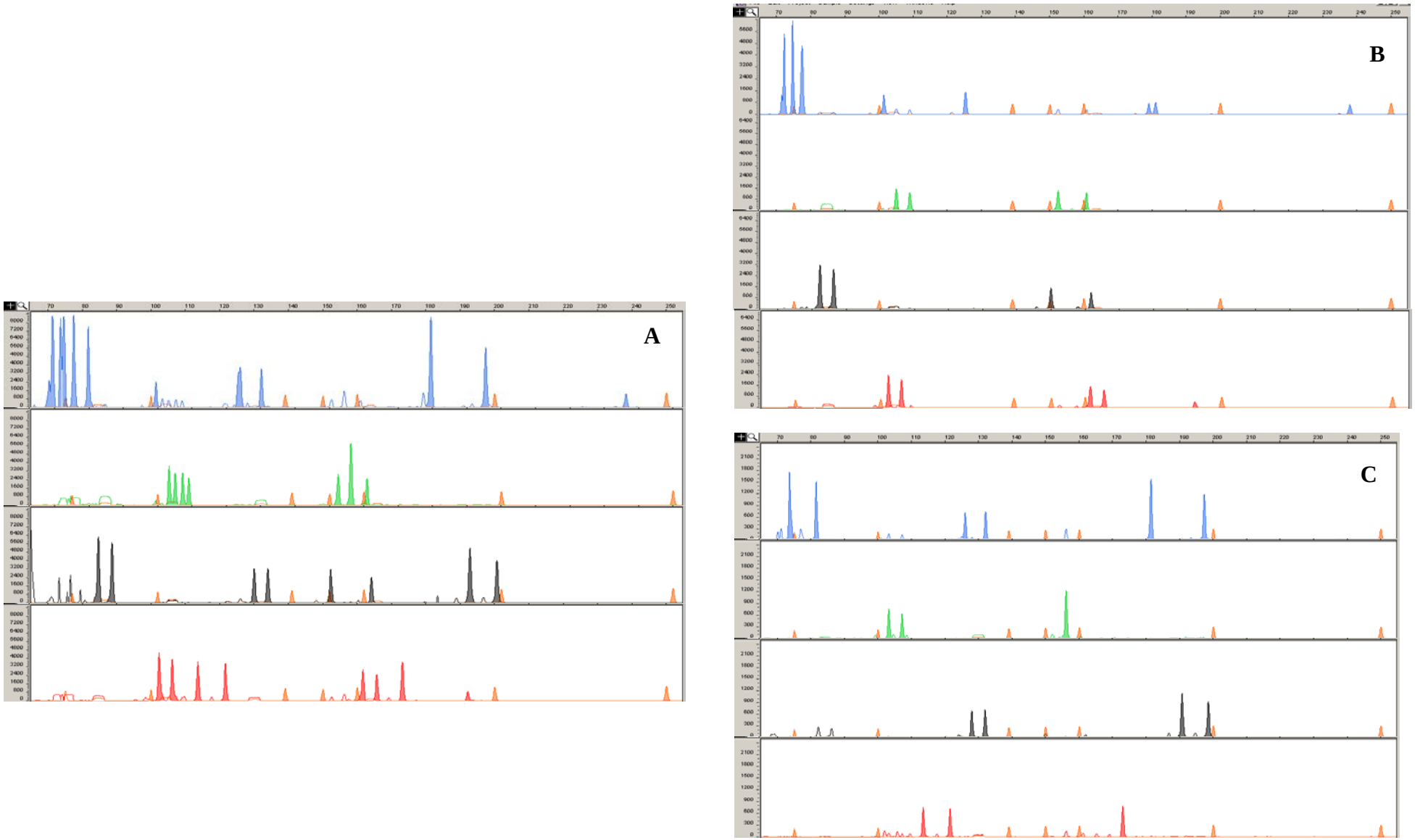


Figure 30. Panel A: Miniplex 1 and 2 comamplified using R60 male genomic DNA. Amplicons overlapped with STR loci with similar size fragments for each fluorescent dyes. **Panel B:** 5 µl of PCR products were aliquoted and Endonuclease Venzyme were added and only PCR products generated by Miniplex 1 was genotyped. **Panel C: 5** µl of PCR products were genotyped and only PCR products generated by Miniplex 2 were genotyped.

produced the desired Miniplex 1 or Miniplex 2 DNA profiles. An example is shown in Figure 30. The DNA profiles generated were characterised with numerous spectral bleed-throughs as a matrix to deconvolute each fo the dye colours used in the forward primers for the multiplexing were not available and thus were not performed.

Futher validation would be required in order to apply Miniplex C to casework samples. For a start, a matrix or spectral calibration would need to be performed on the genetic analyser system before further validation tests, similar to this study that was performed for Miniplex 1 and 2 would be required. The feasiblity tests that were done, indicated the viablity of this novel approach. The success of which meant that more genotype results can be obtained from highly degraded DNA samples and genotyping more amplicons with identical sizes in one amplification attempt.

10. Conclusion

This study was performed using the validation guidelines as specified by SWGDAM. Miniplex 1 and 2 contain all CODIS markers in addition to D2S1338, D19S433 and D2S1776 and gender specific markers, SRY, DYS390 and DYS392. The approach allowed an effective tool for the analysis of degraded DNA samples and also highly reliable gender identification. The developmental validation study demonstrates the robustness, sensitivity, stability and reproducibility of the miniplex assays. For Miniplex 1, where PCR inhibitors are present in high concentration, caution should be exercised during interpretation. The studies did demonstrate that Miniplex 2 would provide reliable genotyping under inhibitory conditions. Perhaps fewer STR loci in a multiplex could be

utilized to reduce the number of primer to primer interaction, using the same PCR buffer and enzyme combinations to prevent non-specific amplifications in inhibitory conditions. Alternative primers could also be tested. Another approach could be the use of other DNA polymerases that have similar properties as OmniTaq being reported (Hedman *et al.* 2009). The studies however did show the use of OmniTaq and EzWay Direct PCR buffer to be extremely robust in overcoming amplification inhibitors and would still be a good PCR system to consider with other primer sets such as the non-codis multiplex that our laboratory has developed.

Recommendations for routine laboratory process were also established as a result of this study, where using 250 – 500 pg of DNA with 30 cycles of PCR would produce complete DNA profiles with good peak balance. Allele concordance studies demonstrated that D7S820 and D16S539, would require additional degenerate primers to offset the primer mutations found in the Malay and Indian population groups would need to be addressed. These studies provided important data for our laboratory to assess the suitability of the Miniplex sets under a variety of conditions that may arise in the interpretation of data from complex forensic samples. Further tests could be conducted to non-routine forensic samples such as hair, fingernail scrapings, bone-extracted DNA samples. As both miniplex assays are shown to work well with degraded and low copy number samples, it is anticipated using these primer sets for these samples would improve genotyping success rates.

Miniplex C demonstrated a novel concept of forensic DNA typing approach. This approach, when fully validated would mean that future genotyping of limited quantity of degraded DNA would yield more STR genotype in one given amplification attempt. The

initial success indicated that this approach should be futher evaluated in order to achieve a highly desirable outcome in forensic DNA typing.

In conclusion, this project and the validation tests done showed that Miniplex 2 is a robust and reliable tool which can provide an alternative to standard STR typing kits under inhibitory conditions and when allele drop out and low sensitivity of large amplicons becomes a problem due to DNA degradation. For Miniplex 1, the application to high inhibited samples must be interpreted with caution in view of the non-specific amplification that was encountered during the validation studies. The success of this project has enabled our laboratory to obtain useful genotype information from what would be otherwise samples that are un-amplifiable under our existing technology. This new approach could be extended to other forensic DNA laboratories as Miniplex C has shown great potential in charting a new strategy towards how forensic DNA typing would be performed in the near future.

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